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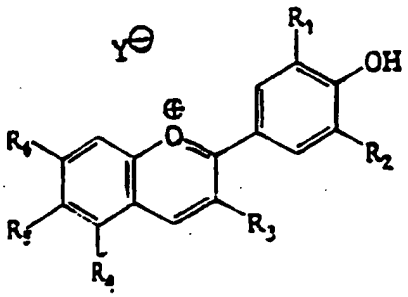

(I)

(57) Abstract

The invention relates to the use of an anthocyanidin or an anthocyanidin derivative of general formula (I) wherein R_1, R_2, R_3 and R_6 independently of each other is H, OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups, R_4 is OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups, R_5 is H, OH, and Y is a counterion or a salt, prodrug, a chemical modification or complex thereof for the preparation of a pharmaceutical composition for the prevention and/or treatment of neoplastic disorders, diseases caused by lesions in connective tissues or a disease caused by a virus in a mammal including a primate such as a human, as well as to novel anthocyanin derivatives of general formula (I) and methods for preparation of said compounds, novel pharmaceutical compositions and methods of treating retroviral infections.

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USE OF ANTHOCYANIDIN AND ANTHOCYANIDIN DERIVATIVES

The present invention relates to the use of an anthocyanidin or an anthocyanidin derivative of the general formula I or a pharmaceutically acceptable salt, prodrug or complex thereof for the preparation of a pharmaceutical composition for the prevention and/or treatment of neoplastic disorders, diseases caused by lesions in connective tissues or a disease caused by a virus in a mammal including a primate such as a human.

BACKGROUND OF THE INVENTICN

Anthocyanins are the most important group of water-soluble plant pigments visible to the human eye. As the anthocyanins seem to have non-toxic effects on the human being, their possible pharmaceutical use has been further investigated.

In PCT/NO95/00185 the present inventors have disclosed that an anthocyanidin or an anthocyanidine derivative is useful for the prevention or treatment of a disease caused by a retrovirus such as, e.g., HIV-1 and HIV-2. The inventors contemplate that the anthocyanidin or anthocyanidin derivatives inhibits the reverse transcriptase or HIV integrase encoded by human immunodeficiency virus (HIV) type 1 (HIV-1) and type 2 (HIV-2). Based upon further experimentation, the present inventors have now found that anthocyanindin or anthocyanidin derivatives and pharmaceutically acceptable salts thereof exhibit very

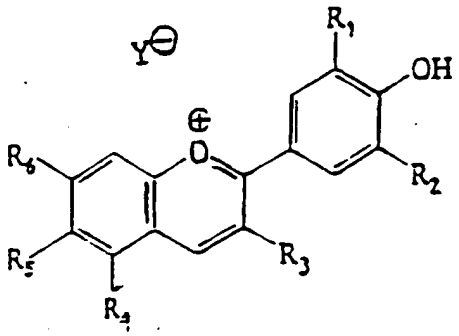
promising effect also against other types of viruses than retrovirus and against neoplastic disorders and diseases caused by lesions in connective tissues.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses that anthocyanidin and anthocyanidin derivatives can exhibit antiviral effects in infected cells and that they exhibit antineoplastic effects in neoplastic cells. Further, the present invention discloses that anthocyanidin and anthocyanidin derivatives can inhibit the degradation of the extracellular matrix and connective tissues. A very important feature by the anthocyanidins and anthocyanidin derivatives is that it has been found that the anthocyanidins and anthocyanidin derivatives are substantially harmless to mammalian cells in concentrations at which they effectively exert the antineoplastic or antiviral effect. This selectivity is very surprising.

In the present context the term "anthocyanidin" denotes an aglycone of an anthocyanin and the term "anthocyanidin derivative" denotes any derivative of an anthocyanidin including any anthocyanin as well as a derivative of an anthocyanin and a derivative of an aglycone of an anthocyanin (i.e. a derivative of an anthocyanidin).

The present invention relates to the use of an anthocyanidin or an anthocyanidin derivative of the general formula I



wherein

R_1, R_2, R_3 and R_6 independently of each other are H, OH, C_{1-6}-alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_4 is OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_5 is H, OH, and

Y is a counterion,

or a prodrug, a chemical modification or complex thereof for the preparation of a pharmaceutical composition for the prevention and/or treatment of neoplastic disorders,

diseases caused by lesions in connective tissues or a disease caused by a virus in a mammal including a primate such as a human.

In particular, the invention relates to the use of a compound wherein at least one of R_1, R_2, R_3, R_4, and R_6 is an -O-glycosyl group, an -O-glycosyl group which is substituted with at least one acyl group, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups. The -O-glycosyl moiety may comprise at least two glycosyl groups and at least one acyl group arranged alternate with one glycosyl followed by one acyl group; an acyl group may also be located at the very end of the moiety.

Several anthocyanins are commercially available. However, many of these anthocyanins can undergo decomposition in a medium having a pH in a range corresponding to the physiological pH range (i.e. about 5-9) and may therefore prove to be difficult to present as a stable pharmaceutical composition. The present inventors have found that more stable anthocyanins are obtained if R_3 in formula I is an -O-glycosyl group which is substituted with at least one acyl group, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups. The -O-glycosyl moiety may comprise at least two glycosyl groups and at least one acyl group arranged alternate with one glycosyl followed by one acyl

group; an acyl group may also be located at the very end of the moiety. Therefore, the invention also relates to the use of a compound wherein R_3 is as defined above.

The compounds of formula I can also be chemically modified by known methods for instance to increase the stabillity of the compounds. Therefore, the invention also relates to the use of a compound of formula I which is chemically modified to increase the stability.

A presently preferred embodiment of the invention is the use of the compound petanin wherein, with reference to formula I, R_1 is OCH_3,

R_2 is OH,

R_3 is 6-O-(4-*O*-*E*-*p*-coumaroyl-a-L-rhamnopyranosyl)-b-D-glucopyranosyl,

R_4 is b-D-glucopyranosyl,

R_5 is H,

and R_6 is OH.

Other presently preferred embodiments are the use of the individual anthocyanins outlined in Tables I and II below, e.g. in compositions wherein the relative quantities of the various anthocyanins are as outlined in Table I or Table II.

TABLE I

Structures and relative proportions (%) of the individual anthocyanins in the first purified *Vaccinium myrtillus* sample (sample VA-1) with reference to formula I, R_4 is OH, R_5 is H and R_6 is OH

COMPOUND	R_1	R_2	R_3	Proportions (%)
1. Delphinidin-3-galactoside	OH	OH	-O-galactosyl	10.6
2. Delphinidin-3-glucoside	OH	OH	-O-glucosyl	10.7
3. Cyanidin-3-galactoside	OH	H	-O-galactosyl	6.8
4. Delphinidin-3-arabinoside	OH	OH	-O-arabinosyl	10.6
5. Cyanidin-3-glucoside	OH	H	-O-glucosyl	8.1
6. Petunidin-3-galactoside	OCH_3	OH	-O-galactosyl	4.6
7. Cyanidin-3-arabinoside	OH	H	-O-arabinosyl	*
8. Petunidin-3-arabinoside	OCH_3	OH	-O-glucosyl	15.2*
9. Peonidin-3-galactoside	OCH_3	H	-O-galactosyl	1.2
10. Petunidin-3-arabinoside	OCH_3	OH	-O-arabinosyl	2.9
11. Peonidin-3-glucoside	OCH_3	H	-O-glucosyl	**
12. Malvidin-3-galactoside	OCH_3	OCH_3	-O-galactosyl	10.3**
13. Malvidin-3-glucoside	OCH_3	OCH_3	-O-glucosyl	14.1
14. Peonidin-3-arabinoside	OCH_3	H	-O-arabinosyl	0.9
15. Malvidin-3-arabinoside	OCH_3	OCH_3	-O-arabinosyl	4.0

* Pigment 7 and 8 together
** Pigment 11 and 12 together

TABLE II

Structures and relative proportions (%) of the individual anthocyanins in the second purified *Vaccinium myrtillus* sample (sample VA-2) with reference to formula I, R_4 is OH, R_5 is H and R_6 is OH

COMPOUND	R_1	R_2	R_3	Proportions (%)
1. Delphinidin-3-galactoside	OH	OH	-O-galactosyl	6.4
2. Delphinidin-3-glucoside	OH	OH	-O-glucosyl	7.4
3. Cyanidin-3-galactoside	OH	H	-O-galactosyl	20.2
4. Delphinidin-3-arabinoside	OH	OH	-O-arabinosyl	11.0
5. Cyanidin-3-glucoside	OH	H	-O-glucosyl	22.8
6. Petunidin-3-galactoside	OCH_3	OH	-O-galactosyl	1.6
7. Cyanidin-3-arabinoside	OH	H	-O-arabinosyl	*
8. Petunidin-3-arabinoside	OCH_3	OH	-O-glucosyl	11.4*
9. Peonidin-3-galactoside	OCH_3	H	-O-galactosyl	1.2
10. Petunidin-3-arabinoside	OCH_3	OH	-O-arabinosyl	**
11. Peonidin-3-glucoside	OCH_3	H	-O-glucosyl	**
12. Malvidin-3-galactoside	OCH_3	OCH_3	-O-galactosyl	8.5**
13. Malvidin-3-glucoside	OCH_3	OCH_3	-O-glucosyl	7.4
14. Peonidin-3-arabinoside	OCH_3	H	-O-arabinosyl	trace
15. Malvidin-3-arabinoside	OCH_3	OCH_3	-O-arabinosyl	2.1

* Pigment 7 and 8 together
** Pigment 10, 11 and 12 together

The compound or mixture of compounds for use according to the invention are claimed to be potent antineoplastic candidates while they at the same time exhibit a very low toxic effect on normal cells and normal cell growth. Thus, the compound or mixture of compounds for use as antineoplastic compounds may be further defined as an anthocyanidin or an anthocyanidin derivative, which, when dissolved in DMSO at a concentration so that the final concentration of DMSO does not exceed 0.2% v/v DMSO, and tested as described in section 2.3, does not have a cytotoxic effect on the growth of uninfected SupT1 cells resulting in a decrease in OD_{580} of more than 50% such as, e.g., 40%, 30%, 20%, or 10% as a result of incubation with the anthocyanidin or the anthocyanidin derivative, and when tested according to a standard test system for testing potential anticancer drugs demonstrates an antineoplastic effect. Such a standard test could be, e.g., a systematic protocol established by the National Cancer Institute (NCI) involving the testing of a compound against a standard cell line panel containing 60 human tumor cell lines. The protocol and the established statistical means for analyzing the results obtained by the standardized testing are well described in the literature, see, e.g., Boyd M. R.: Principles & Practice of Oncology, PPO Updates, Volume 3, No. 10, October 1989 (description of the testing protocol) and Paul, K.D.: "Display and Analysis of Patterns of Differential Activity of Drugs Against Humor Tumor Cell Lines, Development of Mean Graph and COMPARE Algorithm, Journal of the National Cancer Institute Reports, Vol. 81, No. 14, p. 1088, July 14, 1989 (description of the methods of statistical analysis).

In analogy, the compound or mixture of compounds for use as inhibitors of the degradation of conective tissues may be further defined as an anthocyanidin or an anthocyanidin derivative, which, when dissolved in DMSO at a concentration so that the final concentration of DMSO does not exceed 0.2% v/v DMSO, and tested as described in section 2.3, does not have a cytotoxic effect on the growth of uninfected SupT1 cells resulting in a decrease in OD_{580} of more than 50% such as, e.g., 40%, 30%, 20%, or 10% as a result of incubation with the anthocyanidin or the anthocyanidin derivative, and when tested on various proteinases, and especially on matrix metalloproteinases (MMPs) exhibit an inhibiting effect.

In analogy, the compound or mixture of compounds for use as antiviral compounds may be further defined as an anthocyanidin or an anthocyanidin derivative, which, when dissolved in DMSO at a concentration so that the final concentration of DMSO does not exceed 0.2% v/v DMSO, and tested as described in section 2.3, does not have a cytotoxic effect on the growth of uninfected SupT1 cells resulting in a decrease in OD_{580} of more than 50% such as, e.g., 40%, 30%, 20%, or 10% as a result of incubation with the anthocyanidin or the anthocyanidin derivative, and when tested in a standard virus test system shows antiviral effect.

It is contemplated that the anthocyanidin or anthocyanidin derivatives and pharmaceutically acceptable salts thereof are effective against viruses selected from the group consisting of: parvovira; papovavira, such as papilloma virus; andenovira; herpesvira such as Epstein-Barr virus, cytomegalovirus, herpes

simplex vira (HSV 1 and HSV 2), varicella, herpex zoster virus, hepatitis A, hepatitis B; poxvira such as vaccinia, smallpox, molluscum contagiosum, cowpox, and monkey pox virus; hepadnavira; picornavira such as rhinovira and enterovira; reovira such as rotavirus and orbivirus; arbovira such as toga-, flavi-, bunya-, rhabdo-, arena-, and reovira; coronavira; leukaemia, and sarcoma vira; orthomyxovira such as influenza vira; paramyxovira such as mumps virus, measles virus, parainfluenza virus, and RSV; and other unclassified viruses such as lentivira, non-A,non-B hepatitis vira, and viroids.

Furthermore, it is contemplated that the anthocyanidin or anthocyanidin derivatives and pharmaceutically acceptable salts thereof are effective in the treatment or prevention of neoplastic disorders such as neoplastic disorders selected from the group consisting of epithelial neoplasms and non-epithelial and mixed neoplasms. In the table given below is listed relevant neoplasm based on a histogenetic classification.

Tabel III

Cell or Tissue Type	Benign	Malignant
Epithelial neoplasms surface	papilloma	carcinoma soft carcinoma, cirrous carcinioma hard carcinoma, squamous-cell carcinoma, basal-cell carcinoma, ransitional cell carcinoma, capillary carcinoma, apudomas, esidiocytoma, clear-cell carcinoma,choriocarcinoma, and trabecular carcinoma

Cell or Tissue Type	Benign	Malignant
glandular	adenoma	adenocarcinoma spheroidal cell carcinoma, cystadenocarcinoma, papillary adenocarcinoma, and mucous or colloid carcinoma
Non-epithelial and mixed neoplasms		
Connective tissues		
adipose	lipoma	liposarcoma
fibrous	fibroma	fibrosarcoma
cartilage	chondroma	chondrosarcoma
bone	osteoma	osteosarcoma
smooth muscle	leiomyoma	leiomyosarcoma
striped muscle	rhabdomyoma	rhabonyosarcoma
mesothelia		mesothelioma
Neuro-ectodermal		
glial cells	-	gliomas, astrocytoma, oligodendroglioma, ependymoma and anaplastic variants
nerve cells	ganglioneuroma	neuroblastoma medulloblastoma retinoblastoma
melanocytes	pigmented naevus	malignant melanoma
meninges	meningioma	malignant meningioma
nerve sheaths	schwannoma neurofibroma	neurofibrosarcoma
Haemopoietic and lymphoreticular		leukaemias acute leukaemias, monocytic leukaemia, myeloblastic leukaemia (AML), lymphoblastic leukaemia (ALL) and chronic leukaemia, chronic mycloid leukaemia (CML),

Cell or Tissue Type	Benign	Malignant
		chronic lymphocytic leukaemia (CLL), hairy cell leukaemia other myeloproliferative disorders, myelomatosis, myelofibrosis lymphomas Hodgkin's disease, non-Hodgkin's lymphomas, and histiocytic lymphomas
Blood vessels and lymphatic vessels	haemangioma glomangioma lymphangioma	haemangiosarcoma Kaposi's disease lymphangiosarcoma
Germinal and embryonal cells	benign teratoma	malignant teratoma dysgerminoma (F) serminoma (M)
placenta	hydatidiform mole	choriocarcinoma

The integrity of connective tissues is determined by the balance of resorption and repair of components of their cellular matrix. The activity of proteolytic enzymes is rate-limiting for the degradation and therefore the resorption of the collagen and other macromolecular constituents of the extracellular matrix. Among the potential proteinases, the matrix metalloproteinases (MMPs) have a major role in physical resorption of collagen and other macromolecules in development and postnatal remodelling and in pathological resorption associated, for example, with local invasiveness of malignant tumours, resorption of the periodontal structures in periodontal disease, and the destruction of joints in rheumatoid

arthritis. The MMP genes are among the most abundant of those expressed by cells in these inflammatory and malignant lesions.

Thus, it is anticipated that inhibitors of MMPs, especially MMP-1, inhibit tumor invasion and metastasis, and also control the activity of MMPs and preserve the integrity of the extracellular matrix, allowing the extracellular matrix to maintain its control over neoplastic progression. Further, it is anticipated that inhibitors of MMP-1 will inhibit the degradation of connective tissues.

Based upon the disclosure of the present invention, a person skilled in the art will be able to test the compounds of formula I as outlined. Substances which are considered useful may then be tested for cytotoxic effects in other appropriate cell systems such as different fibroblasts (e.g. HeLa) or other uninfected T-cell lines, uninfected T-cell (e.g. cell lines from ATCC), and in primary human lymphocytes from blood donors, e.g. enriched for CD4+ cells by means of Dynabeads®. Furthermore, toxicity tests may be performed such as single dose toxicity tests, e.g. LD_{50} (i.e. the dosage at which half of the experimental animals die). In addition to the LD_{50} value in rodents it is desirable to determine the highest tolerated dose and/or lowest lethal dose for other species, e.g. dog and rabbit. If the *in vitro* test results are promising and the LD_{50} is high, clinical experiments using humans may be approved taking into consideration the specific type of cancer or virus aimed at.

A person skilled in the art would by use of methods described in standard textbooks, guidelines and regulations as well as common general knowledge within the field be able to select the exact dosage regimen to be implemented for any selected compound using merely routine experimentation procedures.

During the process, the person skilled in the art may decide not to continue studying all the initially selected compounds, or it may be decided to synthesize and test new compounds in view of the initial toxicity and biological results obtained.

The cytotoxic and antiviral effects in HIV infected cells have been examined for one anthocyanin sample isolated from blue potatoes (*Solanum tuberosum*) (Sample SP), which contains one clean anthocyanin (called petanin) comprising an aglycone, three monosaccharide moieties and one aromatic acyl group. The cytotoxic effect is also tested for samples VA-1 and VA-2. Samples VA-1 and VA-2 both contain a mixture of anthocyanins. Each anthocyanin in these mixtures are built from only one aglycone and one monosaccharide. Sample VA-2 contains the same, however, a reduced number of anthocyanin compared to Sample VA-1. Sample SP which contains only one, rather complex anthocyanin, shows the best test results.

Further studies may be performed with respect to other types of viruses and various concentrations of the compounds and mixtures of compounds according to the invention in order to titrate the exact concentration at which a cytotoxic effect or an antiviral effect is obtained.

With reference to Formula I, relevant examples of "C_{1-6} alkoxy" are methoxy, ethoxy, propoxy, isopropoxy, n-butoxy, isobutoxy, tert.butoxy, pentoxy and hexoxy.

In one embodiment of the invention the alkoxy is selected from the group consisting of methoxy, ethoxy, propoxy, isopropoxy, and butoxy, such as R_1, R_2, R_3, and/or R_4 being methoxy. In a presently preferred embodiment of the invention, the anthocyanin or the anthocyanin derivative is derived from an anthocyanidin selected from the group consisting of pelargonidin, apigeninidin, and aurantinidin.

In certain embodiments of the invention at least one of R_1 and R_2 is H, whereas in other embodiments at least one of R_1 and R_2 is OH. In a presently preferred embodiment, the anthocyanin or the anthocyanin derivative is derived from an anthocyanidin selected from the group consisting of cyanidin, delphinidin, luteolinidin, tricetinidin, 6-hydroxy-cyanidin, 6-hydroxy-delphinidin, 5-methyl-cyanidin, and pulchellidin.

In still other embodiments, at least one of R_1 and R_2 is alkoxy. It is presently preferred that in this embodiment the anthocyanin or the anthocyanin derivative is derived from an anthocyanidin selected from the group consisting of peonidin, petunidin, malvidin, rosinidin, europinidin, hirsutidin, and capensinidin.

The glycosyloxy may be selected from the group consisting of mono-, di-, tri-, oligo-, polysaccharides, and derivatives thereof. In particular, the glycosyloxy may be substituted with

one or more acyl groups, or the glycosyl may comprise at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups.

In particular, the acyl group may be selected from the group consisting of acyl groups derived from aromatic and aliphatic acyl groups, such as the group consisting of 4-coumaric acid, caffeic acid, ferulic acid, sinapic acid, 4-hydroxybenzoic acid, gallic acid, acetic acid, oxalic acid, malonic acid, malic acid, maleic acid, and succinic acid.

In one embodiment of the invention, the glycosyl group is a group derived from a monosaccharide selected from the group consisting of glucose, galactose, rhamnose, arabinose, xylose, and glucuronic acid.

In another embodiment, the glycosyl group is a group derived from a disaccharide selected from the group consisting of 1,2-glucosylglucoside (sophorose), 1,3-glucosylglucoside (laminariobiose), 1,6-glucosylglucoside (gentiobiose), 1,2-xylosylgalactoside (lathyrose), 1,2-rhamnosylglucoside (neohesperidose), 1,6-rhamnosylglucoside (rutinose), 1,2-xylosylglucoside (sambubiose), 1,6-arabinosylglucoside, and 1,6-rhamnosylgalactoside.

In a third embodiment, the glycosyl group is a group derived from a trisaccharide selected from the group consisting of 1,2-glucosyl-1,6-glucosylglucoside, 1,2-glucosyl-1,6-rhamno-

sylglucoside, 1,2-xylosyl-1,6-glucosylglucoside, and 1,2-xylosyl-1,6-glucosylgalactoside.

As mentioned in the introduction, anthocyanins are water-soluble glycosides and acylglycosides of anthocyanindins, which are polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium (flavylium cation). They belong to the phenolic class of flavonoids with the typical A-ring benzoyl and B-ring hydroxycinnamoyl systems. There are almost 300 naturally occurring structures. The structure of the naturally occurring anthocyanins can be classified according to the basis structure of the aglycone of the anthocyanin, i.e. the anthocyanidin. The following classification is normally used:

i) common basic structures: pelargonidin (Pg), cyanidin (Cy), and delphinidin (Dp)

ii) common methylated structures: peonidin (Pn), petunidin (Pt), and malvidin (Mv)

iii) rare 3-desoxy structures: apigeninidin (Ap), 6-hydroxy-Cy-(6OHCy), and 6-Hydroxy-Dp(6OHDp)

iv) rare methylated structures: 5-methyl-Cy(5MCy), rosinidin (Rs), pulchellidin (Pl), Europinidin (Eu), Hirsutidin (Hs), and Capensinidin (Cp).

The anthocyanidins and anthocyanidin derivatives which are useful according to the present invention are mainly based on one or more of the structures mentioned above. Especially, anthocyanins are considered as potential antineoplastic and/or antiviral candidates, and also as potential inhibitors of the degradation of the extracellular matrix and connective tissues. The anthocyanins occur as 3-monoglycosides, 3-biosides and 3-triosides as well as 3,5-diglycosides and more rarely 3,7-diglycosides associated with the sugars glucose, galactose, rhamnose, arabinose and xylose. A specific example of an anthocyanins which is a potential candidate according to the invention is as mentioned above petanin. Further interesting anthocyanins are:

Pelargonidin 3-arabinoside
Pelargonidin 3-glucopyranoside
Pelargonidin 3-galactoside
Pelargonidin 3-rhamnoside
Pelargonidin 3-(6''-acetylglucoside)
Pelargonidin 3-(6''-malonylglucoside)
Pelargonidin 3-(6''-malylglucoside)
Pelargonidin 3-(6''-E-caffeylglucoside)
Pelargonidin 3-sophoroside
Pelargonidin 3-neohesperidoside
Pelargonidin 3,5 diglucoside
Pelargonidin 3-galactoside-5-glucoside
Pelargonidin 3-(6''-acetylglucoside)-5-glucoside
Pelargonidin 3-(6''-succinylglucoside)-5-glucoside
Pelargonidin 3-caffeoylglucoside-5-glucoside
Pelargonidin 3-(4-coumaroylglucoside-5-glucoside
Pelargonidin 3-caffeoylglucoside-5-malonylglucoside
Pelargonidin 3,5-di-(6-acetylglucoside)
Pelargonidin 3,5-diglucoside acylated with malonic acid and 4-coumaric acid
Pelargonidin 3-caffeoylglucoside-5-dimalonylglucoside
Pelargonidin 3-feruloylgalactoside-5-glucoside
Pelargonidin 3-(4-coumaroylgalactoside)-5-glucoside
Pelargonidin 3-sophoroside-5-glucoside
Pelargonidin 3-(6-(E-(glucosyl)caffeyl)-glucoside)-5-glucoside
Pelargonidin 3-sophoroside-5-glucoside and its di-caffeoylglucoside
Pelargonidin 3-sophoroside-5-glucoside and its tri-caffeoylglucoside
Pelargonidin 3-rhamnosylgalactoside-5-glucoside
Pelargonidin 3-(4-coumaroylrhamnosylgalactoside)-5-glucoside
Pelargonidin 3-feruloylrhamnosylgalactoside-5-glucoside
Pelargonidin 3-(2-(glucosyl)-6-(E-caffeyl)-glucoside)-5-glucoside

Pelargonidin 3-(2-(6-(E-3-(glucosyl)caffeyl)-glucosyl)-glucoside)-5-glucoside
Pelargonidin 3-(2-(6-(E-3-(glucosyl)caffeyl)-glucosyl)-6-(E-caffeyl)-glucoside)-5-glucoside
Pelargonidin 3-(2-(6-(E-3-(glucosyl)caffeyl)-glucosyl)-6-(E-4-(6-(E-3-(glucosyl)caffeyl)-glucosyl)caffeyl)-glucoside)-5-glucoside
Cyanidin 3-arabinoside
Cyanidin 3-xyloside
Cyanidin 3-glucoside
Cyanidin 3-acetylglucoside
Cyanidin 3-(6''-malonylglucoside)
Cyanidin 3-(6''-oxalylglucoside)
Cyanidin 3-malylglucoside
Cyanidin 3-(2''-galloylglucoside)
Cyanidin 3-[6''-(4-coumaroyl)glucoside]
Cyanidin 3-caffeoylglucoside
Cyanidin 3-(dimalonylglucoside)
Cyanidin 3-galactoside
Cyanidin 3-(2''-galloylgalactoside)
Cyanidin 3-sophoroside
Cyanidin 3-[(6''-malonylglucosyl)glucoside]
Cyanidin 3-gentiobioside
Cyanidin 3-laminaribioside
Cyanidin 3-malonyllaminaribioside
Cyanidin 3-glucosyl(4''-sinapoylglucoside)
Cyanidin 3-sambubioside
Cyanidin 3-(6''-E-p-coumaroyl-2''-xylosyl-glucoside)
Cyanidin 3-neohesperidoside
Cyanidin 3-rutinoside
Cyanidin 3-(4''-acetylrhamnosyl)glucoside
Cyanidin 3-(2''-galloyl-6''-rhamnosylglucoside)
Cyanidin 3-(2''-glucuronylglucoside)
Cyanidin 3-(4''-malonyl-2''-glucuronylglucoside)
Cyanidin 3-(6''-malonyl-2''-glucuronylglucoside)
Cyanidin 3-xylosylgalactoside

Cyanidin 3-glucosylgalactoside
Cyanidin 3-(6-(6-sinapoylglucosyl)-galactoside)
Cyanidin 3-rhamnosylgalactoside
Cyanidin 3,5-diglucoside
Cyanidin 3-(6''-acetylglucoside)-5-glucoside
Cyanidin 3,5-di-(6''-acetyl-glucoside)
Cyanidin 3-[6''-4-coumaroyl)glucoside]-5-glucoside
Cyanidin 3-(6''-caffeoylglucoside)-5-glucoside
Cyanidin 3-[6''(4-coumaroyl)glucoside]-5-glucoside
Cyanidin 3-(6''-feruoylglucoside)-5-glucoside
Cyanidin 3,3'-diglucoside malonylcaffeyl
Cyanidin 3,3'-diglucoside caffeyl
Cyanidin 3-[6''-(4-coumaroyl)glucoside]-5-(6'''-malonylglucoside)
Cyanidin 3-(6''-feruloylglucoside)-5-(6''-malonylglucoside)
Cyanidin 3-[6''-(4-coumaroyl)glucoside]-5-(6'''-malonylglucoside)
Cyanidin 3-(6''-caffeoylglucoside)-5-(6'''-malonylglucoside)
Cyanidin 3-(4-coumaroyl)glucoside-5-dimalonylglucoside
Cyanidin 3-caffeoylglucoside-5-dimalonylglucoside
Cyanidin 3-xylosylglucosylgalactoside
Cyanidin 3-[(6''-sinapoylglucosyl)xylosylgalactoside]
Cyanidin 3-[(6''-(4-coumaroyl)glucosyl)xylosylgalactoside]
Cyanidin 3-[(6''-feruloylglucosyl)-xylosylgalactoside]
Cyanidin 3-[(6''(4-hydroxy-benzoyl)glucosyl)xylosylgalactoside]
Cyanidin 3-sambubioside-5-glucoside
Cyanidin 3-(6''-Z-p-coumaroyl-2''-xylosyl)-glucoside)-5-glucoside
Cyanidin 3-(6''-E-p-coumaroyl-2''-xylosyl)-glucoside)-5-glucoside
Cyanidin 3-sambubioside-5-glucoside acylated with malonic acid, ferulic acid and sinapic acid
Cyanidin 3-(6-p-coumaryl-2-(2-sinapyl-xylosyl)-glucoside)-5-(6-malonylglucoside)
Cyanidin 3-(6-p-caffeyl-2-(2-sinapyl-xylosyl)-glucoside)-5-(6-malonylglucoside)

Cyanidin 3-(6-p-feruloyl-2-(2-sinapoyl-xylosyl)-glucoside)-5-(6-malonylglucoside)

Cyanidin 3-(6-p-feruloyl-2-(2-sinapoyl-xylosyl)-glucoside)-5-(6-glucoside)

Cyanidin 3-diglucoside-5-glucoside

Cyanidin 3-diglucoside-5-glucoside acylated with 4-hydroxybenzoyl

Cyanidin 3-diglucoside-5-glucoside acylated with caffeic acid

Cyanidin 3-diglucoside-5-glucoside acylated with caffeic acid and 4-hydroxybenzoic acid

Cyanidin 3-caffeylferulysophoroside-5-glucoside

Cyanidin 3-sophoroside-5-glucoside acylated with 4-hydroxybenzic acid, 4-coumaric acid, caffeic acid, and ferulic acid

Cyanidin 3-[6''-(4-coumaroyl)glucosylglucoside]-5-glucoside

Cyanidin 3-(6''-feruloylglucosylglucoside)-5-glucoside

Cyanidin 3-(6''-sinapoylglucosylglucoside)-5-glucoside

Cyanidin 3-[6''-(4-coumaroyl)glucosyl-(2'''-sinapoylglucoside)]-5-glucoside

Cyanidin 3-[6''-feruloylglucosyl-[2''-sinapoylglucoside)]-5-glucoside

Cyanidin 3-[(6''-sinapoylglucosyl-(2'''-sinapoylglucoside)]-5-glucoside

Cyanidin 3-trisinapoylglucoside-3',7-diglucoside

Cyanidin 3-sambubioside-5-sophoroside acylated with malonic acid, 4-coumaric acid and sinapic acid

Cyanidin 3-sambubioside-5-sophoroside acylated with malonic acid ferulic acid and sinapic acid

Cyanidin (3-feruloyl,1 x terminal glucose; 3 x feruloyl) 3,7,3'-triglucoside

Cyanidin 3-(6-(4-E-p-coumaryl-rhamnosyl)-glucoside)-5-(6-malonyl-glucoside)-3'-(6-E-caffeyl-glucoside)

Cyanidin 3-(6-malonyl-glucoside)-7-(6-E-p-coumarylglucoside)-3'-(6-(E-4-(6-(E-p-coumaryl-glucosyl)-p-coumaryl-glucoside)

Cyanidin 3-(2-(glucosyl)-6-(trans-4-(glucosyl)caffeyl)-glucosyl)-5-glucoside)
Cyanidin-3-(6-malonyl-glucoside)-7,3'-di-(6-(4-(glucosyl)oxybenzoyl)-glucoside)
Delphinidin 3-arabinoside
Delphinidin 3-glucoside
Delphinidin 3-acetylglucoside
Delphinidin 3-(6''-malonylglucoside)
Delphinidin 3-galactoside
Delphinidin 3-(2''-galloylgalactoside)
Delphinidin 3-rutinoside
Delphinidin 3-neohesperidoside
Delphinidin 3,5-diglucoside
Delphinidin 3-(6''-acetylglucoside)-5-(6'''-acetylglucoside)
Delphinidin 3-(6''-Z-p-coumaroylglucoside)-5-(6'''-malonylglucoside)
Delphinidin 3-galactoside-5-glucoside
Delphinidin 3-(4-coumaroylgalactoside)-5-glucoside
Delphinidin 3-rhamnoside-5-glucoside
Delphinidin 3,5-diglucoside acylated with malonic acid and 4-coumaric acid
Delphinidin 3,5-diglucoside acylated with malonic acid and caffeic acid
Delphinidin 3,5-diglucoside acylated with 4-coumaric acid and 2x malonic acid
Delphinidin 3,5-diglucoside acylated with caffeic acid and 2x caffeic acid
Delphinidin 3-(2''-xylosyl-6''-rhamnosylglucoside).
Delphinidin 3,3',5'-triglucopyranoside
Delphinidin 3-(6''-rhamnosylglucoside)-7-glucoside
Delphinidin 3-rhamnosylgalactoside-5-glucoside
Delphinidin 3-(4-coumaroylrhamnosylgalactoside)-5-glucoside
Delphinidin 3-rutinoside-7-(6-(4-(6-(4-hydroxybenzoyl)-glucosyl)oxybenzoyl-b-D-glucoside)
Delphinidin 3-(6-(trans-4-(6-(trans-3-(glucosyl)-caffeyl)-glucosyl)-caffeyl)-glucoside)-5-((6-malonyl)-glucoside)

Delphinidin 7-(6-(4-(6-(4-hydroxybenzoyl)-glucosyl)-oxybenzoyl)-glucoside-3-(6-rhamnosyl-glucoside)

Delphinidin 7-(6-(4-(6-(4-(6-p-hydroxybenzoyl-glucosyl)oxybenzoyl)-glucosyl)oxybenzoyl)-glucoside)-3-(6-rhamnnosylglucoside)

Delphinidin 7-(6-(4-(6-(4-(glucosyl)oxybenzoyl)-glucosyl)oxybenzoyl)-glucoside)-3-(6-(rhamnosyl)-glucoside)

(6''-(delphinidin 3-(6''-(glucosyl)-glucosyl))) (6''-(apigenin 7-(glucosyl))) malonate

Peonidin 3-arabinoside

Peonidin 3-glucoside

Peonidin 3-acetylglucoside

Peonidin 3-(6''-malonylglucoside)

Peonidin 3-(4-coumaroyl)glucoside

Peonidin 3-galactoside

Peonidin 3-rutinoside

Peonidin 3-sophoroside

Peonidin 3-cinnamoylsophoroside

Peonidin 3-[glucosyl(4''-sinapoylglucoside)]

Peonidin 3-(2''-xylosylgalactoside)

Peonidin 3,5-diglucoside

Peonidin caffeoyl 3,5-diglucoside

Peonidin 3-sophoroside-5-glucoside

Peonidin 3-[6''-4-glucosylcaffeoyl)sophoroside]-5-glucoside

Peonidin 3-sophoroside-5-glucoside and its mono-caffeoylglucoside

Peonidin 3-sophoroside-5-glucoside 6,6''-trans-dicaffeate

Peonidin 3-caffeylferulysophoroside-5-glucoside

Peonidin 3-sophoroside-5-glucoside and its tri-caffeoylglucoside

Peonidin 3-sophoroside-5-glucoside acylated with caffeic acid and 4-hydroxybenzoic acid 4-coumaric acid and ferulic acid

Peonidin 3-sophoroside-5-glucoside 4'-glucosylcaffeate

Peonidin 3-diglucoside-5-glucoside glucoside acylated with 4-hydroxybenzoic acid

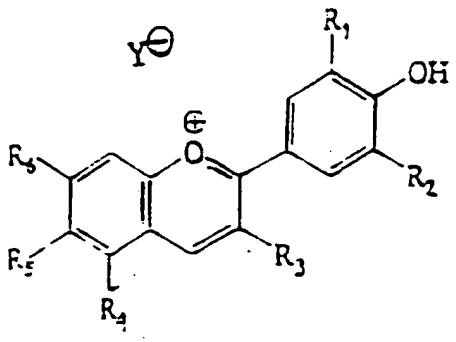
Peonidin 3-(6-(E-(glucosyl)caffeyl)-glucoside)-5-glucoside
Peonidin 3-diglucoside-5-glucoside acylated with ferulic acid
Peonidin 3-diglucoside-5-glucoside acylated with caffeic acid and 4-hydroxybenzoic acid
Peonidin 3-diglucoside-5-glucoside acylated with caffeic acid ferulic acid
Peonidin (3 x feruloyl, 1 x caffeoyl)3-diglucoside-5-glucoside
Peonidin (2 x feruloyl, 1 x caffeoyl)3-diglucoside-5-glucoside
Peonidin 3-(6-(4-E-p-coumaroyl-rhamnosyl)-glucoside)-5-glucoside
Peonidin 3-caffeoylrutinoside-5-glucoside
Petunidin 3-arabinoside
Petunidin 3-glucoside
Petunidin 3-acetylglucoside
Petunidin 3-(6''-malonylglucoside)
Petunidin 3-(4-coumaroyl)glucoside
Petunidin 3-galactoside
Petunidin 3-rutinoside
Petunidin 3-[6''-(4-coumaroyl)rhamnosyl]glucoside
Petunidin 3,5-diglucoside
Petunidin 3-O-(6-O-E-p-coumaroylglucoside)-5-O-(6-O-malonyl-glucoside)
Petunidin 3-rhamnoside-5-glucoside
Petunidin 3-caffeoylrutinoside-5-glucoside
Petunidin 3-O-(6-O-(4-O-E-p-coumaroylrhamnosyl)-glucoside)-5-O-glucoside
Malvidin 3-arabinoside
Malvidin 3-glucoside
Malvidin 3-acetylglucoside
Malvidin 3-(6''-malonylglucoside)
Malvidin 3-(4-coumaroyl)glucoside
Malvidin 3-caffeoylglucoside
Malvidin 3-rutinoside
Malvidin 3,5-diglucoside
Malvidin 3-(6''-malonylglucoside)-5-glucoside
Malvidin 3-(6''-acetylglucoside)-5-glucoside

Malvidin 3-galactoside
Malvidin 3-[6''-(4-coumaroyl)glucoside]-5-glucoside
Malvidin 3,5-diglucoside acylated with 4-coumaric acid and malonic acid
Malvidin 3,5-diglucoside acylated with caffeic acid and malonic acid
Malvidin 3,5-diglucoside acylated with 4-coumaric acid and 2x malonic acid
Malvidin 3,5-diglucoside acylated with caffeic acid and 2x malonic acid
Malvidin 3-rhamnoside-5-glucoside
Malvidin 3-(p-coumarylglucoside)-5-acetylxyloside
Malvidin 3-sophoroside-5-glucoside
Malvidin 3-(dicaffeoylsophoroside)-5-glucoside
Malvidin 3-caffeoylrutinoside-5-glucoside

Other interesting anthocyanins are:
6-Hydroxypelargonidin (aurantinidin)
6-Hydroxycyanidin
6-Hydroxydelphinidin
6-Hydroxycyanidin 3-rutinoside
6-Hydroxydelphinidin 3-rutinoside
Alatanin A
Alatanin B
Alatanin C
Apigeninidin caffeoyl 5-arabinoside
Dimalonylawobanin
Malonylawobanin
Campanin
Cyanodelphin
Heavenly blue anthocyanin
Hyacin
Lobelinin A
Lobelinin B
protodelphin
Monardein
Monodemalonylmonardein

Pelargonidin monodemalonylmonardaein
Nasunin (violanin)
Riccionidin A
Riccionidin B
Rubrocampanin
Salvianin
Monodemalonyl salvianin
Bisdemalonylsalvianin
Salviadelphin
Salviamalvin
Monodemalonylsalviadelphin
Bisdemalonylsalviadelphin
Ternatin A1
Ternatin A2
Ternatin B1
Ternatin D1
Zebrinin

Some of the compounds within the general formula I are known, see e.g. "The Flavonoids", ed. J. B. Harborne, T.J. Mabry and H. Mabry, Chapman & Hall, 1975, "The Flavonoids. Advances in Research", ed. J.B. Harborne and T.J. Mabry, Chapman & Hall, 1982, "The Flavonoids. Advances in Research since 1980", ed. J.B. Harborne, Chapman & Hall, 1988, "The Flavonoids. Advances in Research since 1986", ed. J.B. Harborne, Chapman & Hall, 1994 and references in Chemical Abstract, Vol. 119 to 123 under the General Subject Index entry Anthocyanins. However, the invention in a further aspect relates to novel anthocyanin derivatives of the general formula I



wherein

R_1, R_2, R_3 and R_6 independently of each other are H, OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_4 is OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_5 is H, OH, and

Y is a counterion,

or a prodrug, a chemical modification or complex thereof with the exception of the compounds mentioned above.

Furthermore, the invention relates to a method for the preparation of a novel anthocyanidin or an anthocyanidin derivative of the general formula I as defined above, the method comprising isolation and purification of the anthocyanidin or an anthocyanidin derivative essentially by the method outlined in Example 1. A man skilled in the art will be aware that in isolation and purification of known or novel anthocyanidin and anthocyanidin derivatives, the method described in Example 1 may be amended as appropriate e.g. by use of other extraction procedures and chromatographic techniques.

Alternatively, the compounds which are to be used according to the invention or novel compounds according to the invention may be synthesized e.g. as described in Iacobucci. G.A. and Sweeny, J. G. (1983), "The chemistry of anthocyanins, anthocyanidins and related flavylium salts", *Tetrahedron*, 39, pp. 3005-3038 or as described in Elhabiri, M. et al. (1995), "Anthocyanin chemical synthesis: an important access to natural and synthetic pigments", *Polyphénols Actualités*, No. 13, pp. 11-13. Chemical synthesis of the anthocyanidins and the anthocyanidin derivatives may give appropriate amendments to stabilize the compounds.

In general, anthocyanins from blueberries are rather simple anthocyanins. Compared to other anthocyanins, in particular those acylated with aromatic acids like petanin (sample SP), they are more unstable and may therefore be less useful for pharmaceutical purposes. Thus, forms of anthocyanins involving

co-pigmentation of anthocyanins and intra- and inter-molecular association states of anthocyanins are within the scope of the present invention.

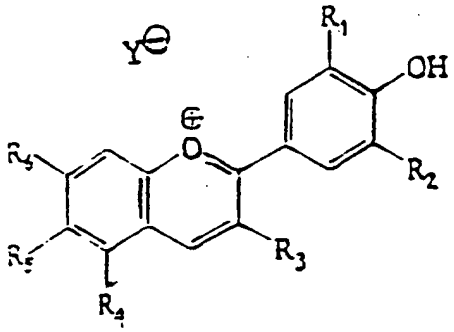
Each anthocyanin may exist in a number of equilibrium forms depending on factors like pH, temperature, concentration, presence of copigments and/or metal ions etc. Together with the variation of building blocks of each anthocyanin and the possibility of existing in several association states (including association with metal ions such as Mg^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+}, other phenolics such as cinnamic acids and other flavonoids, and polymeric material) this allows quite a number of structural modifications which may influence effects/activity. All equilibrium forms and association states are within the scope of the present invention.

As a consequence of asymmetric centres, the compounds of the present invention can occur as mixtures of diastereomers, racemic mixtures and as individual enantiomers. All asymmetric forms, individual isomers and combinations thereof are within the scope of the present invention.

Pharmaceutical compositions comprising mixtures of anthocyanins derived from e.g. blueberries such as Myrtocyan® (*Vaccinium myrtillus* anthocyanosides corresponding to 25% as anthocyanidines) as well as topical medicinal compositions containing fruit juice or fermented fruit juice as described in CA 1086651, a topical composition consisting of an isopropanol extraction of mountain ash berries as described in US 4,132,782, alcoholic extracts of anthocyanosides described in FR 2456747, composi-

tions comprising bilberry anthocyanidines, grape anthocyanidines or elder anthocyanidines described in GB 1,589,294 and anthocyanidin glycosides extracted from bilberries, black currents and blackberries described in US 3,546,337 are known. However, these compositions are based upon partially purified products from fruit or berries and, in addition to the anthocyanin, do also contain other compounds with a potential pharmaceutical activity such as flavonoids. In contrast, the present invention is based upon much more purified anthocyanins.

A further aspect of the invention thus relates to a pharmaceutical composition comprising an anthocyanidin or anthocyanidin derivative of the general formula I



wherein

R_1, R_2, R_3 and R_6 independently of each other are H, OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_4 is OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_5 is H, OH, and

Y is a counterion,

or a prodrug, a chemical modification or complex thereof with the exception of the above mentioned compositions.

A particular preferred embodiment of the invention relates to a pharmaceutical composition comprising petanin in combination with a pharmaceutically acceptable excipient.

Other preferred embodiments are pharmaceutical compositions comprising a mixture of individual anthocyanins as outlined in Table I or in Table II in combination with a pharmaceutically acceptable excipient. Also pharmaceutical compositions comprising a novel anthocyanin derivative in combination with a pharmaceutically acceptable excipient are within the concept of the present invention.

With respect to the counterion Y, it should be recognized that the particular counterion forming part of the salt of this invention is not of a critical nature, as long as it is compatible with the anthocyanidin or anthocyanidin derivative cation.

The counterion is in particular a pharmacologically acceptable anion. The counterion may be organic as well as inorganic in nature.

The term "pharmaceutically acceptable anion" as used herein refers to anions in the salts of the above formula which are substantially non-toxic to living organisms. Typical pharmaceutically acceptable anions include those derived from a mineral or organic acid.

Examples of such inorganic acids are hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, phosphoric acid and the like, and examples of the organic acids are p-toluenesulphonic acid, methanesulfonic acid, oxalic acid, p-bromophenylsulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, acetic acid and the like.

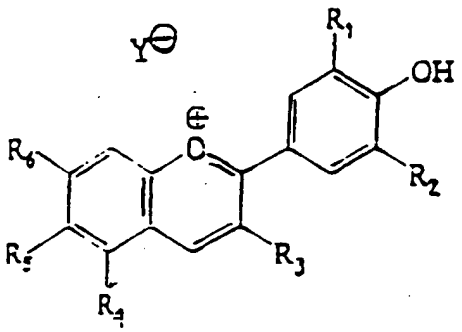
Examples of the anions are sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, proprionate, decanoate, caprylate, acrylate, formate, isobutyrate, caproate, heptanoate, propionate, oxalate, malonate, succinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, sulfonate, xylenesulfonate, phenylacetate, phenylproprionate, phenylbutyrate, citrate, lactate, g-hydroxybutyrate, glycolate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, and mandelate anions, and

the like. Preferred anions are those derived from mineral acids such as hydrochloric acid and hydrobromic acid, and those formed with organic acids such as maleic acid and methanesulfonic acid.

The compositions of the present invention are useful in the prevention or treatment of neoplastic disorders, diseases caused by degradation of connective tissues or a disease caused by a virus.

For these purposes, the compounds of the present invention may be administered orally, parenterally (including subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques), by inhalation spray, or rectally, in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles.

Thus, in accordance with the present invention there is further provided a method for the prevention and/or treatment of neoplastic disorders, diseases caused by lesions in the connective tissues or a disease caused by a virus, the method comprising administering to a mammal in need thereof an effective amount of an anthocyanin derivative of the general formula I



wherein

R_1, R_2, R_3 and R_6 independently of each other are H, OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups

R_4 is OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_5 is H, OH, and

Y is a counterion,

or a prodrug, a chemical modification or complex thereof.

The treatment involves administering to a patient in need of such treatment a pharmaceutical composition comprising a pharmaceutical carrier and a therapeutically effective amount of a

compound of the present invention, or a pharmaceutically acceptable salt thereof.

These pharmaceutical compositions may be in the form of orally administrable suspensions or tablets; nasal sprays; sterile injectable preparations, for example, as sterile injectable aqueous or oleaginous suspensions or suppositories.

When administered orally as a suspension, these compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners/flavouring agents known in the art. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants known in the art.

When administered by nasal aerosol or inhalation, these compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

The injectable solutions or suspensions may be formulated according to known art, using suitable non-toxic, parenterally acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution or isotonic sodium chloride solution, or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid.

When rectally administered in the form of suppositories, these compositions may be prepared by mixing the drug with a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols, which are solid at ordinary temperature but liquidify and/or dissolve in the rectal cavity to release the drug.

Dosage levels of the order of 0.02 to 5.0 or 10.0 g per day are useful in the treatment or prevention of the above-indicated conditions, with oral doses two to five times higher. For example, infection by a virus is effectively treated by the administration of from 1.0 to 50 mg of the compound per kg of body weight from one to four times per day. In one preferred regimen, dosages of 100-400 mg every six hours are administered orally to each patient. It will be understood, however, that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode

and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

The anthocyanidin or anthocyanidin derivatives may be useful either as compounds or mixtures of compounds, pharmaceutically acceptable salts, pharmaceutical composition ingredients, either solely anthocyanidin or anthocyanidin derivatives or in combination with other anti-viral agents, immunomodulators, antibiotics or vaccines. For example, the compounds of this invention may be effectively administered, whether at periods of pre-exposure and/or post-exposure, in combination with effective amounts of other antiviral agents, immunomodulators, anti-infectives, or vaccines known to those of ordinary skill in the art.

In the following Examples, HIV virus has been used in order to demonstrate an effect against retroviruses. It is, however, contemplated that HIV virus can be replaced by other types of non-retroviruses in order to obtain further results.

Experiments in progress to further characterize general biological effects, antineoplastic and antiviral properties, and the connective tissues repairing effect of anthocyanidins or anthocyanidin derivatives

To further study the effect of anthocyanidins in relation to the potentials of these compounds and to get a mechanistic understanding of how these compounds exert their effect a number of different experiments have been initiated or designed.

The experiments can be divided into groups according to the goals of the studies:

I. Cytotoxic effects
   i) In tissue culture studies
   ii) *In vivo* studies in mice

II. *In vitro* studies with defined cell lines

III. Studies on various enzyme systems

IV. Antiviral effects of the compounds measured in tissue culture studies

Studies in group I i) includes characterization of effects of the compounds on cell growth of a number of different established cell lines like the CD4+ human cell lines with lymphocytic phenotypes (Jurkat, CME, H-9, Molt-3, all from ATCC), the monocytic cell line U937 (also from ATCC), and a CD4+ HeLa

(fibroblast) cell line. Peripheral human lymphocytes are also included in these studies. These cells are isolated from normal healthy blood donors, isolated by standard Lymphoprep methods (Nycodens), incubated with the test compounds, stimulated with phytohemagglutinin or cytokines and tested for their ability to incorporate radioactive thymidine.

The aim of these studies is to determine what doses of the test compounds human cells can tolerate without affecting the growth potential of these cells. Furthermore, these studies will be expanded to include long term effects on the cells of low concentrations of the test compounds. At doses where growth is affected, the aim is to study the mechanisms of growth inhibition. To get a general idea of how these compounds interact with cells at toxic or semitoxic doses, the cells are first characterized after treatment with test compounds using electron microscopy. Based on the results of those studies, different biochemical studies will be designed to further elucidate the mechanism behind the cytotoxic effects.

Using these tissue culture systems, pharmacokinetic properties of the compounds will be studied, the goal being to evaluate the efficiency of uptake as well as the stability of the compounds in human cells.

The main goal of the group I ii) studies is to determine LD_{50} in mice. As part of these studies, it is also desired to evaluate the clearance of the different compounds by analyzing urine samples from the treated animals.

The group II studies include a number of standard tests designed to demonstrate whether the test compounds have an antineoplastic effect.

The studies in group III are based on the fact that proteases are digestive enzymes which normally are present in all types of cells within the body (Protein Degradation in Health and Disease, (1980) Ciba Foundation Symposium 75, Excerpta Medica, Amsterdam). During the last years, research within the field of cancer has revealed that neoplastic cell may contain an increased concentration of some proteases compared with the concentration in normal cells (Proteinases and Tumor Invasion, (1980) Monograph Series of the European Organization for Research on Treatment of Cancer, Vol. 6 (Sträuli, P., Barrett, A.J. & Baici, A., eds.), Raven Press, New York). Furthermore, it has been found that neoplastic cells are able to excrete proteases influencing and degrading surrounding cells and tissues (Parish, D.C. (1994) The role of proteolysis in tumour invasion and growth. Endocrine-Related Cancer 1: 19-36). In this way the neoplastic cells get more ready access to growth, propagation and metastases. A relationship between enzymatic activity and metastasis has been found (Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M. & Shafie, S. (1980) Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284: 67-68; Sloane B.F., Dunn. J.R. & Honn, K.Y. (1981) Lysosomal cathepsin B: correlation with metastatic potential. Science 212: 1151-1153).

An enhanced protease activity has been observed in experimental studies of brain tumours (Aardal, N-P., Bjerkvig, R., McDonald, J.K. & Andersen, K-J. (1989) Increased lysosomal exopeptidase activity in brain tumor transition zone. Pathol. Res. Pract. 185:6) and in various types of tumours in thyroidea (Akslen, L.A., Aas, T., Varhaug, J.E. & Andersen, K-J. (1994) Increase of Endo- and Exo-peptidases in Thyroid Tumours. Oncology Reports, 1: 953-956) and in the digestive tract (Aardal, N-P., Solsvik, J. & Andersen, K-J. (1994) The Proteolytic Activity in Normal Mucosa and in Adenocarcinomas of the Colon and Rectum. A Study on 49 Patients. XX International Congress of the International Academy of Pathology, Hong Kong.; Andersen, K-J. & Aardal, N.P. (1996) Acid Hydrolase Activity in Human Colon and Rectum Cancer. In preparation).

Furthermore, it is known that the activity of a number of proteases normally is controlled by specific inhibitors. This may prove to be an important issue in the future treatment of cancer.

The studies in group III are designed to test the effect of anthocyanins on proteases including endo- and exopeptidases. The degree of inhibition of activity is measured in human tissue extracts from normal tissue as well as from various types of tumor tissues. Furthermore, the degree of inhibition of activity is measured in cell extracts from cell cultures of established cell lines and primary cell cultures. In principle the tests can be performed on all proteases; however, the following proteases have been selected for the present purpose:

Aminopeptidases:	Leucine aminopeptidase
	Aminopeptidase M
	Aminopeptidase P
Dipeptidyl peptidases:	Dipeptidyl peptidase I (Cathepsin C)
	Dipeptidyl peptidase II
	Dipeptidyl peptidase IV
Tripeptidyl peptidases:	Tripeptidyl peptidase I (pH 4.5)
	Tripeptidyl peptidase II (pH 7.0)
Endopeptidases:	Cathepsin B
	Cathepsin L
	Cathepsin H
	Tryptase
	Trypsin

The assays are based on fluorescence assay (see e.g. Andersen,K-J. & Ofstad, J. (1986) Adv. Exp. Med. Biol. 198A: 355-359; Andersen, K-J. & Dobrota, M. (1986) Renal Physiol. 9: 275-383; Andersen, K-J., Haga, H.J. & Dobrota, M (1987) Kidney Int. 31: 886-897; Andersen, K-J. & McDonald, J.K. (1987) Am.J.Physiol., 252 (Renal Fluid Electrolyte Physiol. 21): F 890-F 898; Andersen, K-J. & McDonald, J.K. (1987Am. J. Physiol. 253 (Renal Fluid Electrolyte Physiol. 22): F649-F65513-18).

A detailed description of the assays for Cathepsin B, Dipeptidyl peptidase I and Dipeptidyl peptidase II is given in the experimental section.

Effect on cells: The increase in number of cells as a function of time is measured and the usual growth curves are obtained indicating the effect on the ability of the cells to growth and propagation. Assays involving Tryptan blue (the coloured cells indicate dead cells while the uncoloured cells indicate living cells) and Neutral Red Vital Stain are employed (Neutral Red Vital Stain is a convenient chemosensitivity assay for drug screening which is commercially available.

Morphological changes of cells and tissue: The morphological changes are observed and described by means of standard methods for electron microscopy.

Tissue: The enzymatic activity of the above-mentioned enzymes is measured in tumour tissue from patients in order to measure total activity of the individual proteases as well as the inhibitory effect.

Cells from cell cultures:

Primary culture: Cultivation of human tumor tissue from kidney and intestine under standard conditions.

Permanent tumor cell lines: BT4CN and BT4C are employed (both cell lines are glioma cells (brain tumor) from a rat. C6 may also be employed (also glioma cells but a number of normal characteristics from glia cells are retained)

Normal cell lines: LLC-PK1 - kidney cells from proximal tubuli of pigs. MDCK- kidney cells from distal tubili/connecting tubuli of a dog.

IV Based on the results from the group I i) studies, the effect of the test compounds at doses not affecting cell growth on syncytia formation and virus production will be studied in the same cell lines. The aim is to find if there are cell line specificities with respect to the antiviral activities of the compounds studied.

At doses giving an inhibition of viral production, the group IV studies will be conducted. These experiments involve extraction of viral components from infected cells after treatment with the test compounds. The analysis of the extracts include different types of PCR analysis of viral nucleic acids (RNA and DNA) to determine at what stage of the replication cycle inhibition occurs. These studies will be complemented with analysis of viral proteins in the extracts. For the protein analysis, the viral proteins will be metabolically labelled during infection and treatment, precipitated with specific antisera and/or antibodies, and analyzed by SDS-PAGE and autoradiography.

LEGEND TO FIGURES

Figure 1 shows the relationship between cell number and staining by MTT.

Figure 2 shows the effect of DMSO on cell growth and that 0.33% DMSO can be used as a solvent for the compounds without affecting cell growth.

Figure 3 shows the effect of petanin in different concentrations dissolved in DMSO on the growth of SupT1 cells measured after five days of incubation.

Figure 4 shows the effect of the first purified *Vaccinium myrtillus* sample (Sample VA-1) in different concentrations dissolved in DMSO on the growth of SupT1 cells measured after 48 hours of incubation.

Figure 5 shows the effect of the second purified *Vaccinium myrtillus* sample (Sample VA-2) in different concentrations dissolved in DMSO on the growth of SupT1 cells measured after five days of incubation.

Figure 6 shows the effect of petanin (sample SP) in different concentrations on the inhibition of formation of syncytia. The effect is shown as a percentage of the formation of syncytia in cells incubated with only DMSO.

Figure 7 shows the high performance liquid chromatography profiles of the anthocyanin content of *Solanum tuberosum* L during the purification procedure. A, crude extract; B, after partition against ethyl acetate and treatment with Amberlite XAD-7; C, after droplet-current chromatography; D, after Sephadex LH-

gel filtration. The different samples are monitored simultaneously at two different spectral areas (i and ii). The chromatogram labelled B is recorded for the sample SB.

Figure 8 shows the structure of petanin, which is the anthocyanin isolated from *Solanum tuberosum*.

Figure 9 shows the anthocyanin content of the first purified *Vaccinium myrtillus* sample (Sample VA-1) detected at 520 ± 20 nm. The peaks are labelled according to the numbers given in Figure 10.

Figure 10 shows a) the structures and b) the relative proportions (%) of the individual anthocyanins in the first purified *Vaccinium myrtillus* sample (Sample VA-1).

Figure 11 shows the anthocyanin content of the second purified *Vaccinium myrtillus* sample (Sample VA-2) detected at 520 ± 20 nm. The peaks are labelled according to the numbers given in Figure 12.

Figure 12 shows a) the structures and b) the relative proportions (%) of the individual anthocyanins in the second purified *Vaccinium myrtillus* sample (Sample VA-2).

Figure 13 shows inhibition of MMP-1 in extracts from normal and tumor tissues of human.

Figure 14 shows gelatinolytic activity in normal and tumor tissues of rectum of human.

Figure 15 shows gelatinolytic activity in normal and tumor tissues of colon of human.

Figure 16 shows gelatinolytic activity in normal and tumor tissues of ventricle of human.

Figure 17 shows gelatinolytic activity in normal and tumor tissues of pancreas of human.

Figure 18 shows gelatinolytic activity in tumor extracts from rectum preincubated with EDTA (ethylenediaminetetraacetic acid) or EGTA (Ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate).

Figure 19 shows gelatinolytic activity in Tumor extracts from pancreas preincubated with EDTA or EGTA.

Figure 20 shows the effect of various anthocyanidin samples on BT4C and BT4Cn cells.

Figure 21 shows natural red uptake in BT4C cells.

Figure 22 shows natural red uptake in BT4Cn cells.

Figure 23 shows the effect of sample SB on cell number of LLCPK1 and BT4Cn cells exposed to sample SB for 24 hours.

Figure 24 shows prosent of dead LLCPK1 and BT4Cn cells upon exposure to sample SB in 24 hours.

EXAMPLES

TEST METHODS

Determination of cytotoxic and antiviral effects in HIV infected cells of compounds or mixtures of compounds according to the invention

1. Cultivation of cells

The human CD4+ lymphocyte cell line Sup T1 derived from a Non-Hodgkin's T-cell lymphoma patient (Smith et al. (1984), Cancer Research 44, 5657) was a gift from Dr. J. Sodroski at the Division of Human Retroviruses, Dana Farber Cancer Institute, Harvard Medical School, Boston, U.S.A., and was chosen for these studies due to its high content of CD4+ receptors and ability to form large syncytia following infection with HIV-1. The cells were cultivated as suspension cultures in plastic flasks (NUNC, Copenhagen, Denmark - T25 flasks or T125 flasks) in RPMI 1640 medium (Bio Whittaker, Walkersville, MD, USA) supplemented with 5% v/v fetal calf serum, 2 mM glutamine (both from Bio Whittaker) and ABAM (Cat.No. A 9909, Sigma Chem. Company, an 0.1M antibiotic and antimycotic solution containing penicillin and fungizone) in 1 mM final concentration and gentamicine (Bio Whittaker) to a final concentration of 50 mg/ml at 37°C and 5% CO_2 in an incubator (Assab Kebo BioMed).

Counting of cell numbers was performed the same day the experiments started using the Trypan blue exclusion method (Tissue Culture Chemicals, a catalogue from Sigma, 1994) and a Burker counting chamber ("ASSISTENT", Germany) at a magnification of 400x. The ratio between the living and dead cells was at least 95/5 in all experiments determined as described in John Paul, "Cell & Tissue Culture", p. 368, Fifth Edition, Churchill Livingstone, 1975). Prior to the experiments the medium was half-changed in order to add new growth components. The cell density was adjusted to approximately 5 x 10^5 cells/ml and kept at this concentration throughout the experiment by counting the cell number and adding new medium as appropriate or, if necessary, by centrifugation of the cell suspension and resuspension of the cell pellet in an appropriate amount of RPMI 1640 medium.

HIV virus producing Molt 3 IIIB cell line

The cell line was established by infecting Molt 3 cells (American Type Culture Collection, ATCC CRL 1552) with the HIV-1 strain HTLV IIIB obtained from Dr. W. A. Haseltine at the Division of Human Retroviruses, Dana Farber Cancer Institute, Harvard Medical School, Boston, U.S.A. The Molt 3 IIIB cell line is producing virus particles constitutively.

The cells were cultivated as suspension cultures in plastic flasks (NUNC, Copenhagen, Denmark - T25 flasks or T125 flasks) in RPMI 1640 medium (Bio Whittaker, Walkersville, MD, USA) supplemented with 5% v/v fetal calf serum, 2 mM glutamine (both from Bio Whittaker) and ABAM (Cat.No. A 9909, Sigma Chem. Com-

pany, an 0.1M antibiotic and antimycotic solution containing penicillin and fungizone) in 1 mM final concentration and gentamicine (Bio Whittaker) to a final concentration of 50 mg/ml at 37°C and 5% CO_2 in an incubator (Assab Kebo BioMed).

2. Cytotoxicity of the compounds or mixture of compounds tested

2.1. The MTT assay method for determining the number of viable cells

The principle of this assay is based on the cleavage of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazolyl blue, Product No. M 5655, Sigma Chemical Company) to form formazan crystal due to the dehydrogenase activity in the living cells (Mosman, T. et al. J. Immunol. Methods, 65, 55). A standard curve for the MTT assay was established (Fig. 1) by diluting exponentially growing SupT1 cells at known cell numbers in a standard medium (RPMI 1640) into a 96 wells tissue plate (NUNC) at a total volume of 100 ml followed by adding 50 ml of MTT reagent (3 mg/ml in phosphate buffer solution (PBS), pH 7.20) to each well. After addition of the MTT reagent, the plate was incubated at 37°C and 5% CO_2 for 3 hours in an incubator (Assab Kebo BioMed). Then the cells were centrifuged at 2,000 rpm (800 x g) for 10 minutes in a centrifuge equipped with micro-titer plate holders (Beckman centrifuge, GS-6). After centrifugation 100 ml of supernatant was removed from the wells. For this purpose a multichannel micro-pipette was used (Finnpipettes, Finland). The pelleted cells were resuspended in 100 ml DMSO

(dimethyl sulfoxide, Merck) and the plates were gently shaken by hand for about 10 minutes at room temperature before the absorption was read in an ELISA reader (Titerek® Multiskan Plus MK II photometer equipped with a 580 nm light filter (Flow Laboratories, USA). The standard curve of the relationship between cell number and staining by MTT is shown in Figure 1 with a ranging from 10^3 cells/well to 5 x 10^4 cells/well corresponding to OD_{580} = 0.01 and OD_{580} = 0.50, respectively. As shown in Figure 1, within the amount of cells used, there is a linear relationship between the number of living cells and the intensity of staining between cell numbers of 20.000 and 60.000. A new standard curve is established as appropriate e.g. when a new series of experiments are started by a hitherto unexperienced person. The reproducibility of the standard curve is good.

2.2. Determination of the effect of DMSO on cell growth

Since the water solubility of the compounds to be tested varies, the compound or mixture of compounds to be tested are dissolved in DMSO prior to addition to the cell cultures. The effect of DMSO on the cell growth was therefore tested. The cells were added to a 96 wells micro-titer plate; each well containing 1 x 10^4 cells in 100 ml of RPMI 1640 medium. To the suspension of cells was then added DMSO at different concentrations ranging from 0.01% v/v DMSO to 1.0% v/v DMSO. Following incubation in an incubator (Assab Kebo BioMed) at 37°C with 5% CO_2, the amount of living cells as a function of the DMSO concentration was evaluated after 1, 2, and 5 days of incubation by applying the MTT assay as described above. From

the results of the experiments (Figure 2) it can be deducted that the maximum concentration of DMSO that could be used without affecting cell growth was 0.2% v/v. Above that concentration DMSO has a significant effect on the growth of SupT1 cells. At 0.2% v/v concentration or lower of DMSO, practically no difference between cells with or without DMSO could be observed. For this reason compounds to be tested in SupT1 cultures in the presence of DMSO have to be kept in solutions at concentrations so that the final concentration of DMSO does not exceed 0.2% v/v DMSO.

2.3. Effect of test substances on the growth of uninfected SupT1 cells

For each substance to be tested or for each mixture of substances to be tested, 3 parallel experiments were done. Survival of cells were tested after 1, 2, 5, and 7 days, respectively, after starting treatment of the cells with the substances. The cells were maintained in a 96 wells micro-titer plate. To each well 1 x 10^4 cells in 100 ml RPMI 1640 medium were added. To the suspension of cells was then added 10 ml of the test substance in DMSO and RPMI 1640 medium in order to ensure that the final concentration of DMSO did not exceed 0.2% v/v. As control, cells with or without DMSO were used. At the end of incubation at 37°C and 5% CO_2 for 3 hours in an incubator (Assab Kebo BioMed)) with the compounds, survival of the cells was measured by adding the MTT reagent and the samples were processed as described above in section 2.2.

The results of the 3 extracts containing 3 different compounds or mixture of compounds dissolved in DMSO after two to five days of incubation are shown in Figures 3-5.

3. Testing compounds or mixture of compounds for antiviral effects

The screening of antiviral effect of different compounds or mixtures of compounds was based on measuring the formation of syncytia as the exact number of syncytia present after infection of cells with HIV-1 can easily be counted by use of an inverse microscope and thereby an effect obtained by the compound or mixture of compounds added can be measured.

HIV-1 containing supernatant from Molt 3 IIIB cell supernatant was prepared by centrifugation of the Molt 3 IIIB cell culture at 1,000 rpm in a Beckmann GS-6 centrifuge equipped with a GH-3.7 rotor for 5 minutes. In order to standardize the supernatant with respect to the amount of virus, p24 Ag was measured using an ELISA based technique (Sundqvist et al. (1989), J. Medical Virology 29:170-175).

Each virus supernatant used in the experiment had a p24 Ag concentration of 1.5 - 2 ng/10^5 cells. Each T25 (NUNC) flask was filled with 1 x 10^4 cells/ml in a total volume of 5 ml. The test substances was added 30 minutes prior to the addition of the virus containing supernatant and during this preincubation the flasks were kept at 37°C and 5% CO_2 in an incubator (Assab Kebo BioMed). After preincubation, 500 ml of virus supernatant was added. The number of syncytia was counted after 24 and 48 hours

of incubation at 37°C and 5% CO in an incubator (Assab Kebo BioMed) (this time was found to be the standard times for optimal syncytia formation for this cell line at the concentration of virus used).

For each test substance 2 flasks were used and the syncytia were counted by counting the number of syncytia at 5 different places on each flask in an inverse microscope (Olympus CK 2) using a magnification of 10´, thus giving 10 independent countings for each test substance. The parallels obtained were within +- 10% for each experiment.

The results of petanin (sample SP) are shown in Figure 6. The inhibition of formation of syncytia is shown as a percentage of the formation of syncytia in untreated cells.

4. Determination of antineoplastic effect

Cells tested:

The continuous cancer cell lines BT4C and BT4Cn, both obtained from fetal rat brain cells following *in vitro* transformation after *in vivo* exposure to N-ethyl-N-nitrosurea (Laerum, O.D.., Rajewsky, M.F., Schachner, M., Stavrou, D., Haglid, K.G., & Haugen, Å. (1977) Phenotypic properties of neoplastic cell lines developed from fetal rat brain cells in culture after exposure to ethylnitrosurea in vivo. Z. Krebsforsch. 89: 273-295.), have been studied.

Cell culture:

Cell cultures were routinely maintained at 37°C at 100% relative humidity in an atmosphere of air containing 5% CO_2 in a serum-supplemented medium consisting of Eagle-Dulbecco's Modified Medium with 10% new-born calf serum (Gibco, Grand Island, N.Y.) and four times the prescribed concentration of nonessential amino acids, 2% L-glutamine, penicillin (100 IU/mL), and streptomycin (100 mg/mL). Routinely a total of 10^5 cells were seeded into 25 cm^2 tissue culture flasks (Nunc, Denmark) and confluency was reached after 3-4 days.

1. Cell counting and viability assay:

To obtain the total cell count, cells were trypsinized and collected by centrifugation. Cells were counted electronically using a Coulter counter (Coulter Electronics, Miami, FL). Cell viability was examined using the standard trypan blue dye exclusion test (Freshny, R.I. (1987) culture of animal cells. A manual of basic techniques. Second dition, pp. 245-256. Alan R. Liss, inc. New York).

2. Cytotoxicity assay:

The neutral red vital stain assay for chemosensitivity was used. The assay is based on neutral red as a vital stain accumulates in the lysosomal compartment of the cells following uptake via non-ionic diffusion (Nemes, Z., Dietz, R., Luth, J.B., gomba, S., Hackenthal, F. & Gross, F. (1979) The pharmacological relevance of vital staining with

neutral red. Experientia 35: 1475-1476.; Allison, A.C. & Young, M.R. (1969) Vital staining in flouroscence microscopy of lysosomes. In Dingle, J.T. & Fell, H.B. (eds), Lysosomes in Biology and Pathology, Vol. 2, 600-626. Nemes, Z. et al., 1979 and Allison, A.C. et al., 1969). The assay protocol was: BT4Cn and BT4C cells were seeded in 24-well (16-mm diameter) multidishes from Costar (Cambridge, MA) at a density of 10^4 cells per well and grown in the presence of serum-supplemented medium at 37°C and 5% CO_2 for 24 hours. Then the medium was changed to a chemically defined medium where 5 mg/ml insulin, 20 nM hydro-cortisone, 0.3 nM triiodo-thyronine, 1 mg/ml transferrin, 1.36 mg/ml vitamin B12, 0.007 mg/ml Biotin, 10 mg/ml DL-a-tocopherol, 5 mg/ml retinol, 0.2 mg/ml lipoic acid, and 0.1 mg/ml linoleic acid were used as a substitute for serum (Akslen, L.A.., Andersen, K-J. & Bjerkvik, R., (1988) Characteristics of human and rat glioma cells grown in a defined medium. Anticancer Research 797-804) for 24 hours, before cells were exposed to SA or SB over night at 37°C and 5% CO_2. The cell medium was then decanted and the cells were exposed to 100 ml/well of 0.1% neutral red solution in PBS w/o calcium and magnesium (Gibco) for 3 hours at 37°C. Cells were rinsed with buffer saline and allowed to dry. The dye was eluted with 100 ml of a solution containing 50% ethanol and 1% glacial acetic acid and the absorbance for each well was determined at 540 nm. Background was determined using the same procedure without exposing cells to the neutral red solution.

Assays

CATHEPSIN B

Substrate: 10 mM N-a-CBZ-L-Arginyl-L-Arginine-b-naphtylamine 3 AcOH (Mw: 787,9) (glass 300) 0,029 g/5ml N'N-dimethylformamide

Buffer: 50 mM Phosphate buffer, pH=6,5 with 10mM EDTA 10mM dithiothreitol and 0.1% Triton-X-100:
4,45 g $Na_2HPO_4 2H_2O$
0.1% Triton-X-100
1,86 g EDTA-Titriplex-Na diluted to 500ml with H_2O
0,771 g dithiothreitol

Quenching reagent: 50 mM Glycin buffer pH=10.4 37.6 g glycin/L

With tissue blank		Without tissue blank
Working solution		Working solution
Substrate diluted 1:2 with buffer		Substrate diluted 1:2 with buffer
Assay		Assay
Sample	Tissue blank	Sample
100 ml sample 200 ml buffer Preincubate 5 min 50 ml working solution	100 ml sample 200 ml buffer Preincubate 5 min 50 ml working solution Incubate 15 min	100 ml sample 200 ml buffer Preincubate 5 min 50 ml working solution Incubate 45 min
Incubate 15 min		
2 ml quenching reagent	2 ml quenching reagent	2 ml quenching reagent

Standard: 10 mM b-naphtylamine (Mw 143.2) (Sigma - N7750)
14.3 mg/10 ml dried methoxyethanol

Calculations are performed based on standard curves (excitation wavelength 340 nm, emission wavelength 410 nm)

DDP I (Dipeptidylpeptidase)

Substrate: 10 mM H-Gly-arg-bNa (Mw 392.9) (Glass 101)
0.020 g/5 ml N'N-dimethylformamide

Buffer: 10 mM cacodylate buffer, pH=6,0 (uses cacodylic acid or Na cacodylate) with 10 mM mercaptoethylamine mw=113.6
Mercaptoethylamine is added immediately before use

Quenching reagent: 50 mM Glycin buffer pH=10.4 37.6 g glycin/L

With tissue blank		Without tissue blank
Working solution		Working solution
Substrate diluted 1:2 with buffer		Substrate diluted 1:2 with buffer
Assay		Assay
Sample	Tissue blank	Sample
100 ml sample 200 ml buffer Preincubated 5 min 50 ml working solution Incubate 30 min	100 ml sample 200 ml buffer Preincubated 5 min Incubate 30 min	100 ml sample 200 ml buffer Preincubated 5 min 50 ml working solution
		Incubate 30 min
2 ml quenching reagent	2 ml quenching reagent 50 ml working solution	2 ml quenching reagent

Standard: 10 mM b-naphtylamine (Mw 143.2) (Sigma - N7750)
14.3 mg/10 ml dried methoxyethanol

Calculations are performed based on standard curves (excitation wavelength 340 nm, emission wavelength 410 nm)

DDP II (Dipeptidylpeptidase)

Substrate: 15 mM H-lys-ala-bNa, mw = 342,5, 0.026 g/5ml solved in N.N. dimethylformamide

Buffer: 0.1 phosphate buffer pH = 5.0 with 0.1% Triton-X-100:
1.67 ml orto-phosphoric acid/250 ml + Triton-X-100

Quenching reagent: 50 mM Glycin buffer pH=10.4
37.6 g glycin/L pH adjusted with NaOH

With tissue blank		Without tissue blank
Working solution		Working solution
Substrate diluted 1:2 with buffer		Substrate diluted 1:2 with buffer
Assay		Assay
Sample	Tissue blank	Sample
100 ml sample 200 ml buffer 50 ml working solution	100 ml sample 200 ml buffer	100 ml sample 200 ml buffer 50 ml working solution
Incubate 30 min	Incubate 30 min	Incubate 30 min
2 ml quenching reagent	2 ml quenching reagent 50 ml working solution	2 ml quenching reagent

Standard: 10 mM b-naphtylamine (Sigma - N7750)

Calculations are performed based on standard curves (excitation wavelength 340 nm, emission wavelength 410 nm)

Matrix Metalloproteinase-1 (MMP-1)

in extracts from tumour and normal tissues from cancer patients.

MMP-1 was determined by the Biotrak™ ELISA assay system (code RPN 2610) provided by Amersham International, UK.

COMPONENTS OF THE ASSAY SYSTEM

Microtitre plate

The plate contains 12 x 8 well strips coated with mouse anti-MMP-1. Ready for use.

Assay buffer 1

Bottle contains 10 ml of phosphate buffer concentrate which when diluted gives a 0.1 M phosphate buffer pH 7.5 containing 0.9% (w/v) sodium chloride and 0.1% (w/v) bovine serum albumin and 0.1% Tween ™ 20. This reagent is for dilution of donkey anti-rabbit peroxidase conjugate only.

Assay buffer 2

Bottle contains 10 ml of phosphate buffer concentrate which when diluted gives a 0.1M phosphate buffer pH 7.5 containing 0.9%(w/v) sodium chloride and 0.1%(w/v) bovine serum albumin. This reagent is for dilution of standard, antiserum and samples.

Standard

Vial contains 1 ml human MMP-1 frozen in assay buffer 2 at a concentration of 200 ng/ml. Ready for use after thawing.

Antibody

Bottle contains lyophilised rabbit anti-MMP-1 which on reconstitution gives rabbit anti-MMP-1 in 0.1 M phosphate buffer pH 7.5 containing 0.9% (w/v) sodium chloride and 0.1%(w/v) bovine serum albumin.

Peroxidase conjugate

Bottle contains lyophilised donkey anti-rabbit horseradish peroxidase which on reconstitution gives donkey anti-rabbit horseradish peroxidase in 0.1M phosphate buffer pH 7.5 containing 0.9%(w/v) sodium chloride, 0.1% bovine serum albumin and 0.1% Tween 20.

Wash buffer

Bottle contains 12.5 ml phosphate buffer concentrate which on dilution gives a 0.0067M phosphate buffer pH 7.5 containing 0.033% Tween 20.

TMB substrate

Bottle contains 3,3',5,5'-tetramethylbenzidine (TMB)/hydrogen peroxide, in dimethylformamide, (20%, v/v) 22 ml, ready for use.

Gelatinolytic activity (Gelatinases) in extracts from tumour and normal tissue from cancer patients.

Assay principle: The assay was performed as a standard gelatin zymographic assay. The principles of the assay is based on electrophoretic separation of tissue extracts on

polyacrylamide gels containing gelatin. Following electrophoretic separation the gels are incubated at 37 °C overnight and stained for protein. Zones of gelatinolytic activity are then observed as clear, unstained bands on the gel.

Assay protocol: Gelatine 300 bloom (Sigma Chemical Co.) was added to the standard Laemmli acrylamide polymerisation mixture (11% Stock Resolving solution) at a final concentration of 3 mg/ml. Polymerisation of gels were obtained after 30 min at room temperature. Stacking gel (4% Stacking gel) was polymerised on top of the main gel (30 min room temperature).

Human tissue samples were homogenised (10 mg tissue/ml buffer) in 0.15 M NaCl, pH 7.0, containing 0.1 % Triton X-100, mixed (1:3), before 30 ml were loaded (without boiling and b-mercaptoethanol) into each well of the stacking gel mounted in a BioRad mini-Slab gel apparatus.

Gels were run at 10 mA/gel during resolving phase at 4 °C. After Electrophoresis, gels were soaked in 2.5% Triton-X-100 for 2 hours at room temperature with gentle shaking to elute SDS. The gels were then rinsed, and incubated overnight at 37 °C in a 0.05M Tris-HCl , 0.1 M NaCl, pH 7.6, buffer.

Following incubation the gels were stained for 30 min. in 0.5% Coomassie Blue R-250 in Ethanol:acetic acic:water (30:10:60), destained in Ethanol:acetic acic:water (30:10:60) and photographed.

11% Stock Resolving solution

1	Acrylamide(30%) Bis. (1%) in Water	3,30 ml
2	1,5 M Tris-HCl. PH:8,8 + 0,4% SDS	2,25 ml
3	Gelatine 300 bloom 3g% in distilled water	0,9 ml
4	Ammonium persulphate: 100 mg/ml in water	0,02 ml
5	TEMED (N,N,N',N',-tetramethyl-ethylenediamine)	0,01 ml
6	Water	2,55 ml
	Total Volume	9,03 ml

4% Stacking gel:

1	Acrylamide(30%) Bis. (1%) in Water	0,40 ml
2	0,5 M Tris-HCl. PH:6,8 + 0,4% SDS	0,50 ml
3	Ammonium persulphate: 100 mg/ml in water	0,04 ml
4	TEMED	0,02 ml
5	Water	3,10 ml
6	Total Volume	4,06 ml

Sample Buffer: Without b-mercaptoethanol,

1	Water	4,0 ml
2	0,5 M Tris-HCl. PH:6,8	1,0 ml
3	Glycerol	0,8 ml
4	10% SDS	1,6 ml
5	0,1% Bromphenol.Blue	0,2 ml

Reservoir Buffer: 5X electrophoresis buffer Laemmli method.

1	Tris base	15g/l
2	Glycin	72g/L
3	SDS	5g/L

EXAMPLES

EXAMPLE 1

Isolation and purification of the anthocyanin samples

Samples

PG: pelargonidin 3-O-b-D-glucopyranoside
SA: cyanidin 3-O-b-D-glucopyranoside
SB: petanin (partly purified, see Figures 5 and 6)
SC: cyanidin 3-O-(6''- a-L-rhamnopyranosyl-b-D-glucopyranoside) and delphidinin 3-O-(6''-a-L-rhamnopyranosyl-b-D-glucopyranoside) (content ca. 1:1).
SP: petanin (See Figure 6 for structure)

VA-1 Mixture of anthocyanins from blueberries *(Vaccinium myrtillus* L.)

VA-2: Mixture of anthocyanins from blueberries (*Vaccinium myrtillus* L.)

Extraction

PG

Ripe fruits of strawberry (*Fragaria ananassa* var. Corona) were purchased at the local food marked. The frozen fruits (500 g) were extracted twice for 14 hours in the refrigerator with 500 ml of methanol containing 0.07% v/v concentrated hydrochloric acid.

SA and SC

Ripe fruits of black current (*Ribes nigrum* L.) were collected at Foldøy in Ryfylke on the West coast of Norway. The frozen fruits (450 g) were extracted three times for 14 hours in the refrigerator with 500 ml of methanol containing 0.5% v/v concentrated hydrochloric acid.

SB and SP

Tubers of *Solanum tuberosum* L. (anthocyanin pigmentation in skin and flesh) from cultivation at the Agricultural University of Norway, NLH-Ås, Norway, were cut with a pair of scissors and extracted for 3 hours (three times) with methanol containing 0.1% v/v concentrated hydrochloric acid.

VA-1 and VA-2

Ripe berries of Vaccinium myrtillus L. were collected in Åsane near Bergen on the West coast of Norway. The frozen berries (100 g) were extracted for 5 hours (twice) with 500 ml of methanol containing 0.05% v/v concentrated hydrochloric acid.

Procedures for purification of the samples

For all samples: The filtered extracts were combined and concentrated under reduced pressure at 28°C. The concentrated solutions (ca. 100 ml) were washed twice with ca. 100 ml ethyl acetate, and the lower layers were further concentrated under reduced pressure at 28°C before they were passed through an 18 cm x 2.6 cm Amberlite_ XAD-7 column (an ion exchange resin from BDH Chemicals Ltd.) which had been washed in advance with distilled water. The XAD-7 column (with the adsorbed anthocyanins) was washed with ca. 2 l of distilled water. To elute the anthocyanins, ca. 300 ml each of 50 % aqueous methanol and anhydrous methanol (both containing 0.5% v/v CF_3COOH) were used successively. The samples were then concentrated under reduced pressure at 28°C. The samples containing PG, SA and SC, and SP were individually subjected to droplet counter-current chromatography.

Droplet counter-current chromatography (DCCC)

DCCC was carried out using a Tokyo Rikakikai Eyela Model DCC-300 chromatograph fitted with 300 glass capillaries (40 cm x 2 mm i.d.).

For PG, the lower layer of *n*-butanol-acetic acid-water (4:1:5, v/v) was used as mobile phase. A flow rate of 10 ml/hour were used throughout the experiment. Some stationary phase (100 ml) was displaced prior to elution of the first drop of mobile phase. Then 100 fractions, each of 5 ml, were collected. Fractions 54-100 were collected and concentrated under reduced pressure at 28°C.

For the sample containing both SA and SC, the upper layer of *n*-butanol-acetic acid-water (4:1:5, v/v) was used as mobile phase. A flow rate of 10 ml/hour was used throughout the experiment. Some stationary phase (100 ml) was displaced prior

to elution of the first drop of mobile phase. Then 90 fractions, each of 5 ml, were collected. Fractions 36-42 were collected and concentrated under reduced pressure at 28°C (Sample SA). Fractions 55-90 were collected and concentrated under reduced pressure at 28°C (Sample SC).

For SP, the upper layer of *n*-butanol-acetic acid-water (4:1:5, v/v) was used as mobile phase. A flow rate of 10 ml/hour was used throughout the experiment. Some stationary phase (100 ml) was displaced prior to elution of the first drop of mobile phase. Then 45 fractions, each of 7 ml, were collected. Fractions 12-35 were collected and concentrated under reduced pressure at 28°C.

For VA-1, the lower layer of *n*-butanol-acetic acid-water (4:1:5, v/v) was used as mobile phase. A flow rate of 10 ml/hour were used throughout the experiment. Some stationary phase (150 ml) was displaced prior to elution of the first drop of mobile phase. Then 160 fractions, each of 4 ml, were collected. Fractions 20-100 were collected and concentrated under reduced pressure at 28°C.

For VA-2, the lower layer of *n*-butanol-acetic acid-water (4:1:5, v/v) was used as mobile phase. A flow rate of 9 ml/hour was used throughout the experiment. Some stationary phase (110 ml) was displaced prior to elution of the first drop of mobile phase. Then 150 fractions, each of 4 ml, were collected. Fractions 13-15 were collected and concentrated under reduced pressure at 28°C.

The samples containing PG, SA, SC, SP, VA-1, and VA-2 were individually subjected to Sephadex_ LH-20 chromatography.

Sephadex_ LH-20 chromatography

Sephadex_ LH-20 chromatography was performed on a 100 cm x 3 cm column using 40% v/v aqueous methanol containing 1% v/v CF_3COOH as eluent. All the anthocyanin coloured fractions belonging to each sample were put together and evaporated to dryness under reduced pressure at 28°C.

Monitoring of fractions

Thin-layer chromatography (TLC) analyses were performed on 0.1 mm cellulose layers (Schleicher and Schüll, F1440) in the following solvent systems:

A. Formic acid-concentrated hydrochloric acid-water (5:1:5, v/v)
B. *n*-Butanol-acetic acid-water (4:1:5, v/v, upper phase).

High performance liquid chromatography (HPLC) was carried out using a slurry packed ODS-Hypersil column 20 x 0.5 cm, 5 mm). Two solvents were used for elution (A: formic acid-water (1:9, v/v) and B: formic-acid-water-methanol (1:4:5, v/v). Several slightly different elution profiles were used: A typical elution profile was composed of isocratic elution (90% v/v A, 10% B) over 4 min, linear gradient from 10% v/v B to 100% B over the next 17 min, followed by linear gradient from 100% B to 10% v/v B over 1 min. The flow rate was 1.5 ml min^{-1}, and aliquots of 10 ml were injected.

Determination of relative quantities

The relative quantities of the individual anthocyanins in the purified *Vaccinium myrtillus* samples (VA-1 and VA-2) were based on integration of the different peaks in the HPLC chromatograms (Figure 9 and Figure 11) of the purified samples. These chromatograms were recorded by measuring the absorption values on every second nm between 500 and 540 nm simultaneously, and

do not take into account the different molar absorption coefficients of the individual anthocyanins.

Identification

The identities of the individual anthocyanins were determined by a combination of co-chromatography (HPLC and TLC), degradation techniques, UV-visible spectroscopy, and one- and two-dimensional NMR techniques (Andersen, Ø.M. (1989). Dr. philos.-thesis University of Bergen. ISBN 82-7406-002-4; Andersen, Ø.M. (1988). Acta Chem. Scand. 42, 462; Andersen, Ø.M., Opheim, S., Aksnes, D.W., and Frøystein, N.Å. (1991). Phytochemical Analysis. 2, 230.)

EXAMPLE 2

Determination of cytotoxic and antiviral effects in HIV infected cells in three different extracts containing compounds or mixtures of compounds according to the invention

The cytotoxic effect and the antiviral effect in HIV infected cells of the three extracts obtained according to Example 1 were tested as described in Test Methods, sections 2 and 3.

Cytotoxic effect of a compound or mixture of compounds is defined here as the concentration of the compound or mixture of compounds which effects the growth rate of the cells tested. Here, a cytotoxic effect of a compound or mixture of compounds is considered present if a decrease in OD_{580} of more than 10% is observed as a result of incubation with the compound or mixture

of compounds. With respect to the cytotoxic effect, the results are shown in Figure 3.

An antiviral effect is here considered present if a decrease in syncytia formation of more than 10% is observed as a result of incubation with the compound or mixture of compounds. With respect to the antiviral effect, the results are shown in Figure 4 wherein for each compound or mixture of compounds the inhibition of formation of syncytia is shown as a percentage of the formation of syncytia in untreated cells.

In Figure 6 are shown the results after 24 hours and/or 48 hours. At 48 hours the same pattern is observed although the total amount of syncytia is higher. The compound has a clear inhibitory effect on the cytopathogenic effect of HIV although complete inhibition of syncytia formation cannot be obtained at the experimental conditions used.

I Cytotoxic and antiviral effect of Sample SP

At concentrations between 0.02 and 0.2 mg/ml, a cytotoxic effect on cell growth rate is observed (Figure 3).

At concentrations above 1 mg/ml, an antiviral effect is observed (Figure 6).

EXAMPLE 3

Inhibition of pelargonidin 3-glucoside (Sample PG) on proteolytic enzymes

Pelargonidin 3- glucoside (Pg 3-glc) was tested in tissue extracts from a normal intestinal mucosa from rat. Inhibition is of the proteolytic enzymes is found in a range of about 12-30 % (see the table below). Furthermore, 0.5 mM pelargonidin 3-glucoside has been found to inhibit the uptake of neutral red in LLC-PK1 cells by 16%.

Inhibition of rat control with pelargonidin 3-glucoside (0.23 mM corresponding to 0.1 mg/ml in the incubation solution):

Enzyme	% inhibition
TPP4.5	30.2 ± 2.3
DDP 4	27.0 ± 0.2
DPP 2	20.3 ± 1.1
LAP	31.0 ± 3.1
NAG	12.6 ± 1.2
Cath.H	12.2 ± 4.3
DDP I	21.3 ± 0.8
Cath.B	20.6 ± 1.1

Inhibition of various anthocyanidin samples on Matrix Metalloproteinase-1 (MMP-1)

Typical results obtained for MMP-1 assayed in extracts from normal a tumour tissue are shown in Figure 13 where effects from all compounds tested (Samples SA, SB and SC) are seen as an inhibition of MMP-1 activity in the tumour from

patient no. 3, 4, 7 and 8. Patient no. 1 - 6 are suffering from colon cancer, while patient no. 7 and 8 are suffering from cancer in the rectum.

Determination of Gelationlytic activitiy (Gelatinases) revealed by gelatine zymography

Gelatine-degrading enzymes present in the samples were identified by their ability to clear the substrate (white zones) at their respective molecular weights.

a. Rectum

Typical results from a patient with rectum cancer are shown in Figure 14. Arrows indicate bands revealing gelatinase activity in the tumour extract (white zones) inhibited by SA, SB and SC. Note that the corresponding activity with similar banding in the normal tissue is only inhibited by SB.

b. Colon

Typical results from a patient with colon cancer are shown in Figure 15. Arrows indicate bands revealing gelatinase activity (white zones) in the tumour extract inhibited by SA, SB and SC.

c. Ventricle

Typical results from a patient with ventricle cancer are shown in Figure 16. The arrow indicates gelatinase activity in the tumour extract inhibited by SA, SB and SC.

d. Pancreas

Typical results from a patient with pancreas cancer are shown in Figure 17. Arrows indicate gelatinase activity in the tumour extract inhibited by SA, SB and SC.

Control experiments - tumour specific gelatinases

Because some Matrix Metalloproteinases are known to have gelatinolytic activity, test runs were also performed to see whether the tumour specific gelatinolytic activity demonstrated by gelatine zymography was caused by these enzymes. Matrix Metalloproteinases, as the name implies, are dependent on the presence of divalent metal ions for their activity. Tumour extracts from rectum and pancreas were therefore preincubated with chelating agents such as EDTA and EGTA before gelatine zymography. The results obtained are shown in Figure 18 and Figure 19. Neither EDTA nor EGTA had any effect on the gelatinolytic in the tumour extracts demonstrating that the observed activity are not due to Matrix Metalloproteinase activity.

Conclusion

To our knowledge the tumour specific gelatinolytic activity found for the anthocyanindin samples have not been reported earlier and represents as such new findings.

Example 4

Tests used to demonstrate whether the test compounds have an anti-neoplastic effect as inhibitor of tumour cell metabolism.

Results - cell counting and viability assay

SA and SB were tested at final concentrations up to 1 mM for their effect on BT4C and BT4Cn cell mortality. Figure 20 demonstrate clearly that exposure to both SA and SB increases the mortality from hardly detectable to about 10% in the BT4C cells. In the BT4Cn cells SB gave an increased mortality from 6% to 12%, while no increasing effect was seen from SA at the concentrations tested. Only minor effects were observed on the total cell number after exposure to SA or SB up to a final concentration of 1 mM.

Results - cytotoxicity assay (Neutral red)

The results obtained for cytotoxicity of anthocyanidin derivatives tested on BT4C cells are shown in Figure 21 and on BT4Cn cells are shown in Figure 22. Both compounds were found to reduce neutral red uptake in both cell lines. A similar effect was observed for both SA and SB on BT4C cells where the neutral red uptake was reduced by about 50% by 1 mM SA or SB. A similar effect of about 50% inhibition was seen from SB on BT4Cn cells where SA reduced the uptake by 25-30%.

Morphological observation

Before neutral red was added to BT4C and BT4Cn cells both cells were observed to have accumulated the anthocyanidin derivatives tested (data not shown).

Conclusion:

The Neutral red cytotoxicity assay demonstrate that both SA and SB have cytotoxic effects on the brain tumour cell lines BT4C and BT4Cn. Also the anthocyanidin derivatives tested are taken up by these cell lines.

EXAMPLE 5

LLCPK1 and BT4Cn cells exposed to sample SB

The established renal epithelial cell line LLC-PK1, which has characteristics reminiscent of those of proximal tubular cells (Hull RN, Cherry WR, Weaver GM: The origin and characteristics of a pig kidney strain LLC-PK1. In Vitro 12: 670-677, 1976), has been extensively studied in monolayer cultures.

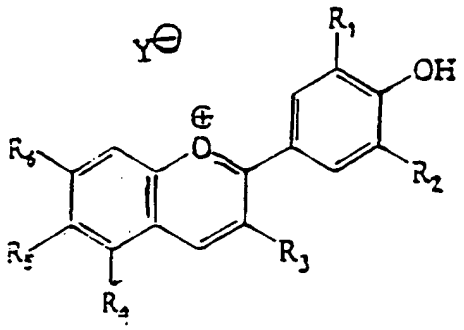
LLC-PK1 cells (CRL 1392; American Type Culture Collection, Rockville, MD, USA) were originally obtained from Flow Laboratories (Irvine, UK) at passage number 202. The cells described in this study were grown from passage number 217-238 in Eagle-Dulbecco's modified medium (Gibco, Grand Island, NY) with 10% new-born calf serum and four times the prescribed concentration of nonessential amino acids, 2% L-

glutamine, penicillin (100 IU/ml) and streptomycin (100 mg/ml). Cell cultures were routinely maintained at 37°C at 100% relative humidity in an atmosphere of 5% CO_2/95% air

A 24 well plate was seeded with BTᵅCn cells and another plate with LLCPK-1 cells at about 30 000 per well. They were grown to semiconfluency, and then exposed to 0, 0.2, 0.4, 0.6, 0.8, 1.0 mM of sample SB for about 24 hours. Then the cells were trypsinized and counted manually for living and dead cells with Tryptan blue, usin Hemocytometer slide.

CLAIMS

1. The use of an anthocyanidin or an anthocyanidin derivative of the general formula I



wherein

R_1, R_2, R_3 and R_6 independently of each other are H, OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_4 is OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_5 is H, OH, and

Y is a counterion,

or a salt, prodrug, a chemical modification or complex thereof for the preparation of a pharmaceutical composition for the prevention and/or treatment of neoplastic disorders, diseases caused by lesions in connective tissues or a disease caused by a virus in a mammal including a primate such as a human.

2. The use according to claim 1, wherein the anthocyanidin or the anthocyanidin derivative, when dissolved in DMSO at a concentration so that the final concentration of DMSO does not exceed 0.2% v/v DMSO, when tested as described in section 2.3, the anthocyanidin or the anthocyanidin derivative does not have a cytotoxic effect on the growth of uninfected SupT1 cells resulting in a decrease in OD_{580} of more than 10% as a result of incubation with the anthocyanidin or the anthocyanidin derivative.

3. The use according to claim 1 or 2, wherein alkoxy is selected from the group consisting of methoxy, ethoxy, propoxy, isopropoxy, and butoxy.

4. The use according to claim 3, wherein R_1, R_2, R_3, and/or R_4 is methoxy.

5. The use according to any of claims 1-4, wherein at least one of R_1 and R_2 is H.

6. The use according to any of claims 1-4, wherein at least one of R_1 and R_2 is OH.

7. The use according to any of claims 1-3, wherein at least one of R_1 and R_2 is alkoxy.

8. The use according to claim 1, wherein the anthocyanin or the anthocyanin derivative is as outlined in claim 1 with the exception of the compounds; pelargonidin, cyanidin, delphinidin and chrysanthemin.

9. The use of an anthocyanidin or an anthocyanidin derivative according to claim 8 for the preparation of a pharmaceutical composition for the prevention and/or treatment of neoplastic disorders.

10. The use according to claim 1, wherein the anthocyanin or the anthocyanin derivative is as outlined in claim 1 with the exception of the compounds; pelargonidin, cyanidin and delphinidin.

11. The use of an anthocyanidin or an anthocyanidin derivative according to claim 8 for the preparation of a pharmaceutical composition for the prevention and/or treatment of diseases caused by lesions in connective tissues.

12. The use according to claim 5, wherein wherein the the anthocyanin or the anthocyanin derivative is derived from an anthocyanidin selected from the group consisting of pelargonidin, apigeninidin, and aurantinidin.

13. The use according to claim 6, wherein the anthocyanin or the anthocyanin derivative is derived from an anthocyanidin selected from the group consisting of cyanidin, delphinidin, luteolinidin, tricetinidin, 6-hydroxy-cyanidin, 6-hydroxy-delphinidin, 5-methyl-cyanidin, and pulchellidin.

14. The use according to claim 7, wherein the anthocyanin or the anthocyanin derivative is derived from an anthocyanidin selected from the group consisting of peonidin, petunidin, malvidin, rosinidin, europinidin, hirsutidin, and capensinidin.

15. The use according to any of the preceding claims, wherein the glycosyloxy is selected from the group consisting of mono-, di-, tri-, oligo-, polysaccharides, and derivatives thereof.

16. The use according to claim 15, wherein the glycosyl group is substituted with one or more acyl groups, or the glycosyl moiety comprises at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups.

17. The use according to claim 16, wherein the acyl group is selected from the group consisting of aromatic and aliphatic acyl groups.

18. The use according to claim 17, wherein the acyl group is selected from the group consisting of acyl groups derived from 4-coumaric acid, caffeic acid, ferulic acid, sinapic acid, 4-hydroxybenzoic acid, gallic acid, acetic acid, oxalic acid, malonic acid, malic acid, maleic acid, and succinic acid.

19. The use according to any one of claims 15-18, wherein the glycosyl group is a group derived from a monosaccharide selected from the group consisting of glucose, galactose, rhamnose, arabinose, xylose, and glucuronic acid.

20. The use according to any one of claims 15-18, wherein the glycosyl group is a group derived from a disaccharide selected from the group consisting of 1,2-glucosylglucoside (sophorose), 1,3-glucosylglucoside (laminariobiose), 1,6-glucosylglucoside (gentiobiose), 1,2-xylosylgalactoside (lathyrose), 1,2-rhamnosylglucoside (neohesperidose), 1,6-rhamnosylglucoside (rutinose), 1,2-xylosylglucoside (sambubiose), 1,6-arabinosylglucoside, and 1,6-rhamnosylgalactoside.

21. The use according to any one of claims 15-18, wherein the glycosyl group is a group derived from a trisaccharide selected from the group consisting of 1,2-glucosyl-1,6-glucosylglucoside, 1,2-glucosyl-1,6-rhamnosylglucoside, 1,2-xylosyl-1,6-glucosylglucoside, and 1,2-xylosyl-1,6-glucosylgalactoside.

22. The use according to claim 1, wherein at least one of R_3, R_4, and R_6 is an -O-glycosyl, an -O-glycosyl group which is substituted with at least one acyl group, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups.

23. The use according to claim 1, wherein

R_1 is OCH_3,

R_2 is OH,

R_3 is 6-O-(4-*O*-*E*-*p*-coumaroyl-a-L-rhamnopyranosyl)-b-D-glucopyranosyl,

R_4 is b-D-glucopyranosyl,

R_5 is H,

and R_6 is OH.

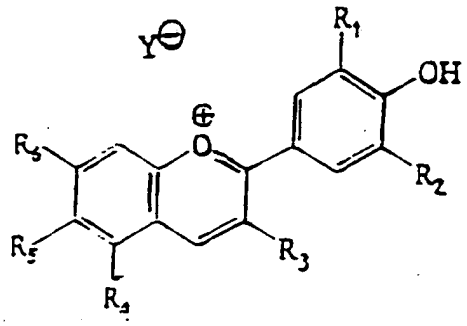
24. The use according to claim 1, wherein the composition consists of the individual anthocyanins outlined in Table I, preferably in the relative quantities outlined in the table.

25. The use according to claim 1, wherein the composition consists of the individual anthocyanins outlined in Table II, preferably in the relative quantities outlined in the table.

26. The use according to any of the preceding claims, wherein the virus is selected from the group consisting of parvovira; papovavira, such as papilloma virus; andenovira; herpesvira such as Epstein-Barr virus, cytomegalovirus, herpes simplex vira (HSV 1 and HSV 2), varicella, herpex zoster virus, hepatitis A, hepatitis B; poxvira such as vaccinia, smallpox, molluscum contagiosum, cowpox, and monkey pox virus; hepadnavira; picornavira such as rhinovira and enterovira; reovira such as rotavirus and orbivirus; arbovira such as toga-, flavi-, bunya-, rhabdo-, arena-, and reovira; coronavira; leukaemia, and sarcoma vira; orthomyxovira such as influenza

vira; paramyxovira such as mumps virus, measles virus, parainfluenza virus, and RSV; and other unclassified viruses such as lentivira, non-A,non-B hepatitis vira, and viroids.

27. A novel anthocyanin derivative of the general formula I



wherein

R_1, R_2, R_3 and R_6 independently of each other are H, OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

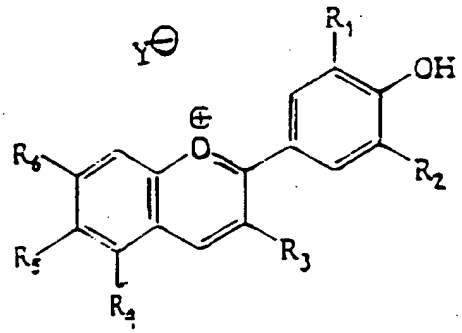
R_4 is OH, alkoxy, an -O-glycosyl, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_5 is H, OH, and

Y is a counterion,

or a prodrug, a chemical modificatin or complex thereof with the exception of the compounds mentioned in "The Flavonoids", ed. J. B. Harborne, T.J. Mabry and H. Mabry, Chapman & Hall, 1975, "The Flavonoids. Advances in Research", ed. J.B. Harborne and T.J. Mabry, Chapman & Hall, 1982, "The Flavonoids. Advances in Research since 1980", ed. J.B. Harborne, Chapman & Hall, 1988, "The Flavonoids. Advances in Research since 1986", ed. J.B. Harborne, Chapman & Hall, 1994 and in references in Chemical Abstract, Vol. 119 and 123 under the General Subject Index entry Anthocyanins.

28. A pharmaceutical composition comprising an anthocyanidin or anthocyanidin derivative of the general formula I



wherein

R_1, R_2, R_3 and R_6 independently of each other are H, OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_4 is OH, alkoxy, an -O-glycosyl, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_5 is H, OH, and

Y is a counterion,

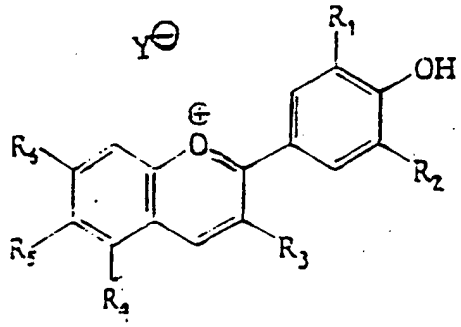
or a prodrug, a chemical modification or complex thereof with the exception of Myrtocyan® (*Vaccinium myrtillus* anthocyanosides corresponding to 25% as anthocyanidines), topical medicinal compositions containing fruit juice or fermented fruit juice as described in CA 1086651, a topical composition consisting of an isopropanol extraction of mountain ash berries as described in US 4,132,782, the alcoholic extracts of anthocyanosides described in FR 2456747, the compositions comprising bilberry anthocyanidines, grape anthocyanidines or elder anthocyanidines described in GB 1,589,294 and the anthocyanidin glycosides extracted from bilberries, black currents and blackberries described in US 3,546,337.

29. A pharmaceutical composition comprising petanin in combination with a pharmaceutically acceptable excipient.

30. A pharmaceutical composition comprising a novel anthocyanin derivative according to claim 27 in combination with a pharmaceutically acceptable excipient.

31. A method for the preparation of an anthocyanidin or an anthocyanidin derivative of the general formula I as defined in claim 27, the method comprising isolation and purification of the anthocyanidin or an anthocyanidin derivative by the methods outlined in Example 1.

32. A method for the prevention and/or treatment of neoplastic disorders, diseases caused by lesions in connective tissues or a disease caused by a virus, the method comprising administering to a mammal in need thereof an effective amount of an anthocyanin derivative of the general formula I



wherein

R_1, R_2, R_3 and R_6 independently of each other are H, OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or more acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_4 is OH, alkoxy, an -O-glycosyl group, an -O-glycosyl group which is substituted with one or one acyl groups, or an -O-glycosyl moiety comprising at least two glycosyl groups and at

least one acyl group arranged so that at least one acyl group is located between two glycosyl groups,

R_5 is H, OH, and

Y is a counterion,

or a prodrug, a chemical modification or complex thereof.

33. A method according to claim 32, wherein the virus is selected from the group consisting of parvovira; papovavira, such as papilloma virus; andenovira; herpesvira such as Epstein-Barr virus, cytomegalovirus, herpes simplex vira (HSV 1 and HSV 2), varicella, herpex zoster virus, hepatitis A, hepatitis B; poxvira such as vaccinia, smallpox, molluscum contagiosum, cowpox, and monkey pox virus; hepadnavira; picornavira such as rhinovira and enterovira; reovira such as rotavirus and orbivirus; arbovira such as toga-, flavi-, bunya-, rhabdo-, arena-, and reovira; coronavira; leukaemia, and sarcoma vira; orthomyxovira such as influenza vira; paramyxovira such as mumps virus, measles virus, parainfluenza virus, and RSV; and other unclassified viruses such as lentivira, non-A,non-B hepatitis vira, and viroids.

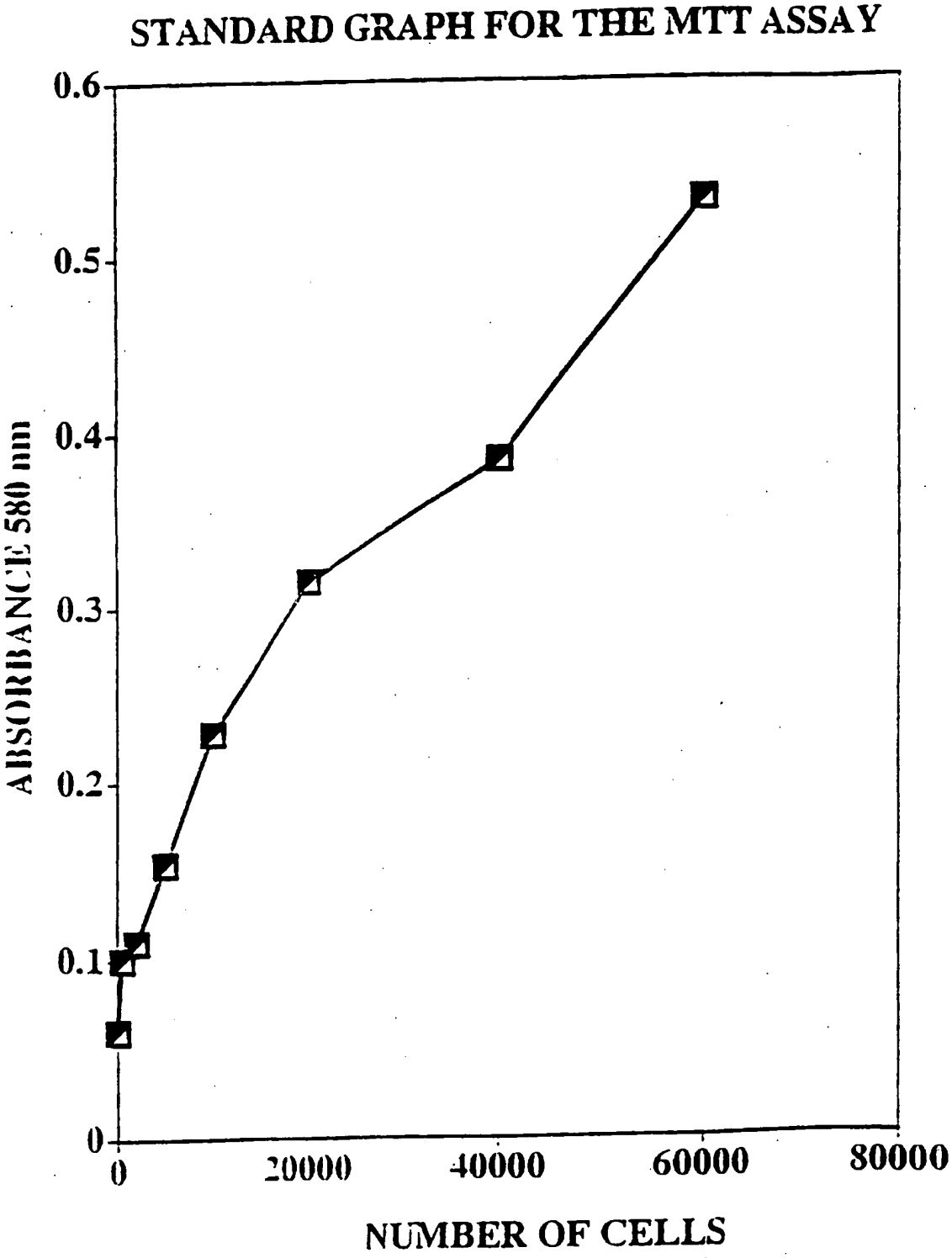

STANDARD GRAPH FOR THE MTT ASSAY
ABSORBANCE 580 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20000
40000
60000
80000
NUMBER OF CELLS

Figure 1

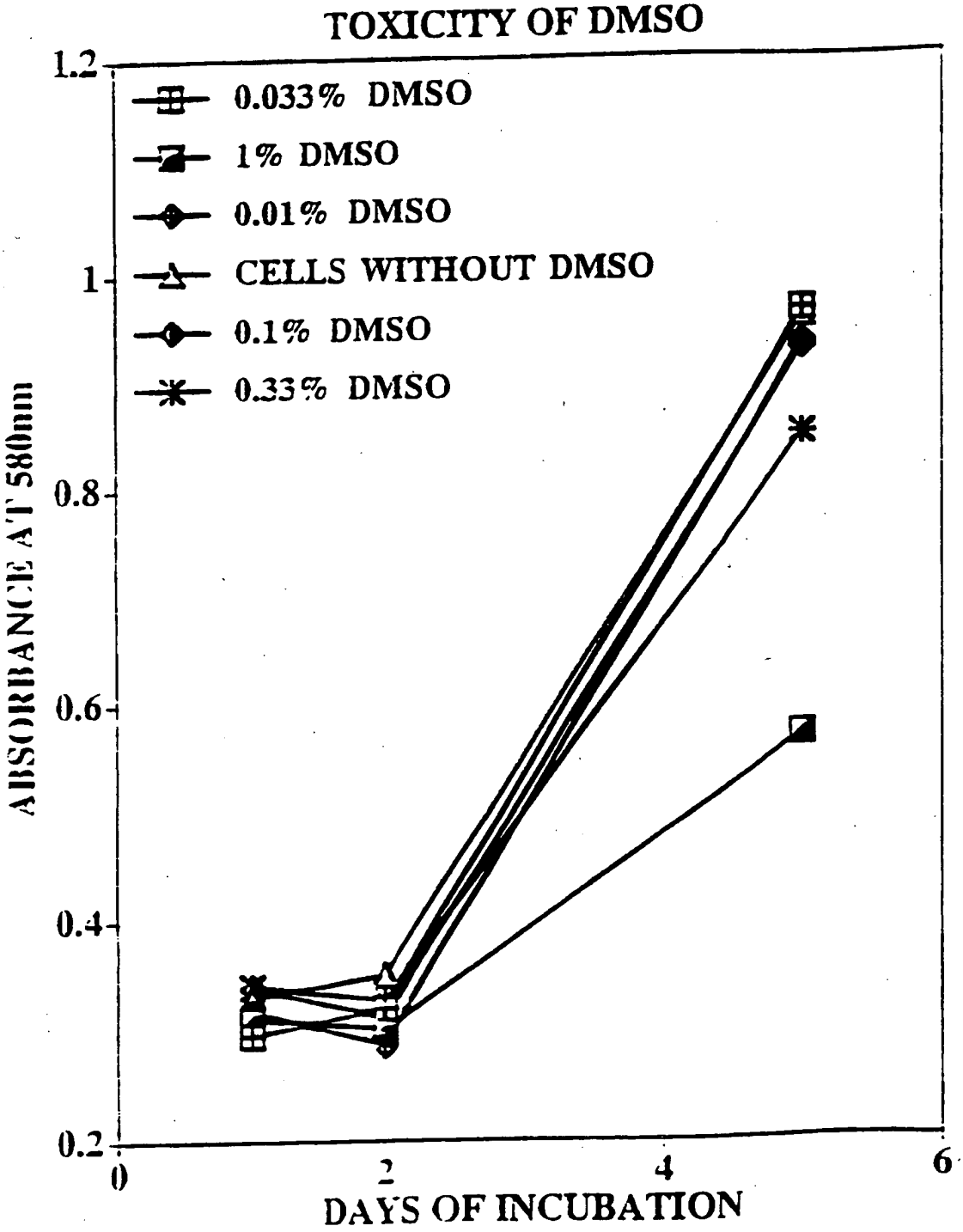

TOXICITY OF DMSO
0.033% DMSO
1% DMSO
0.01% DMSO
CELLS WITHOUT DMSO
0.1% DMSO
0.33% DMSO
ABSORBANCE AT 580nm
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6
DAYS OF INCUBATION

Figure 2

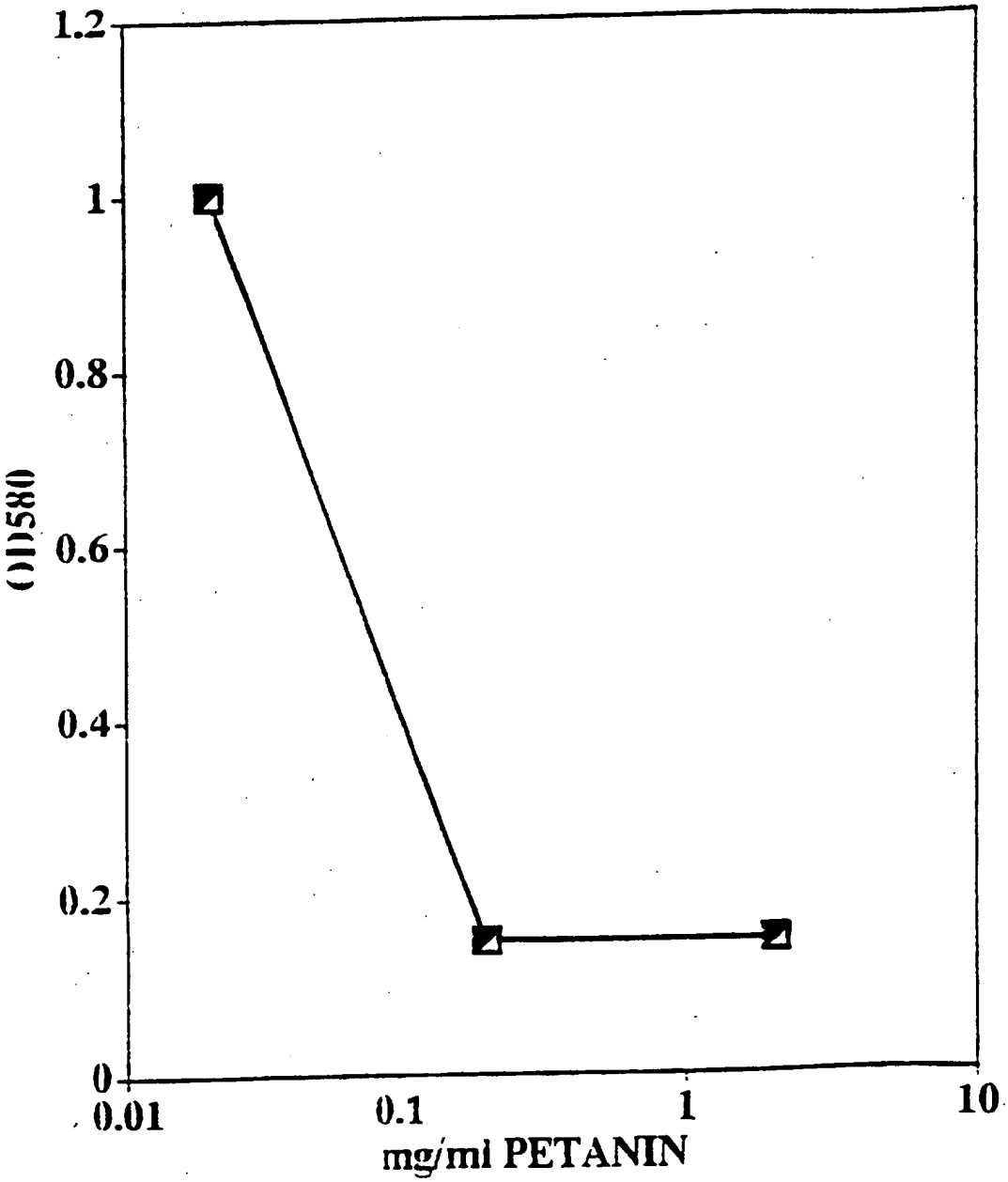


Figure 3

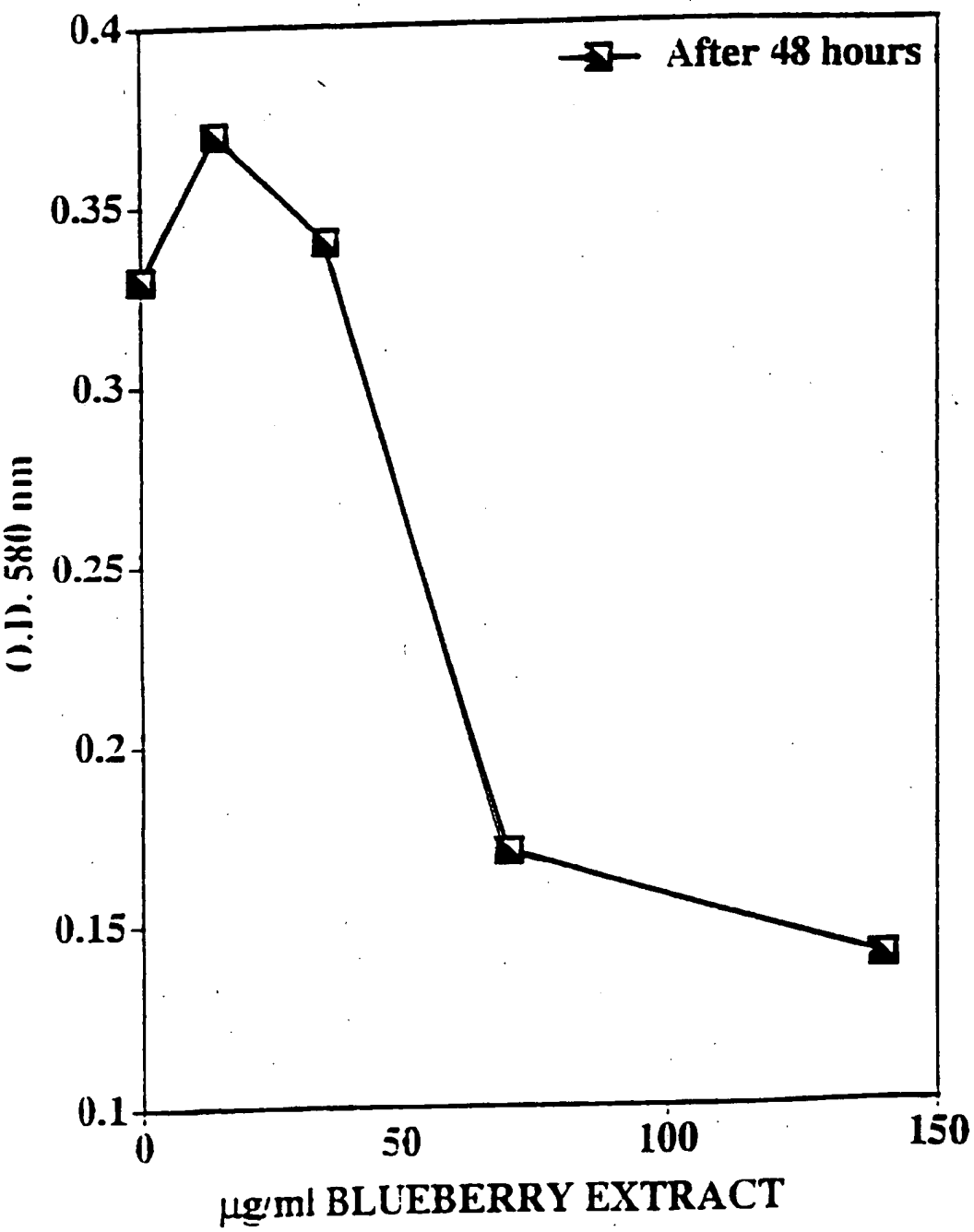

After 48 hours
0.4
0.35
0.3
0.25
0.2
0.15
0.1
O.D. 580 nm
0
50
100
150
µg/ml BLUEBERRY EXTRACT

Figure 4

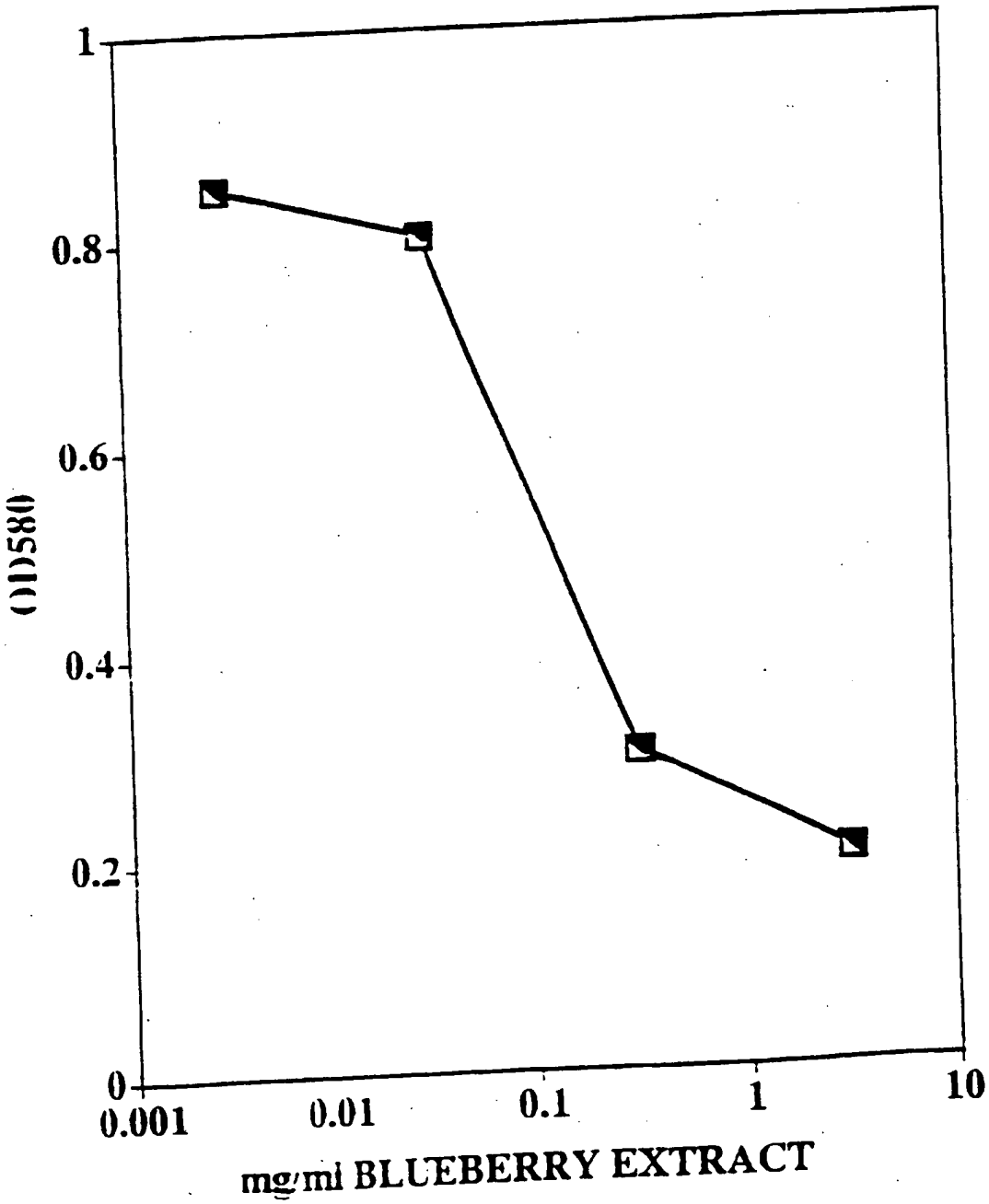


Figure 5

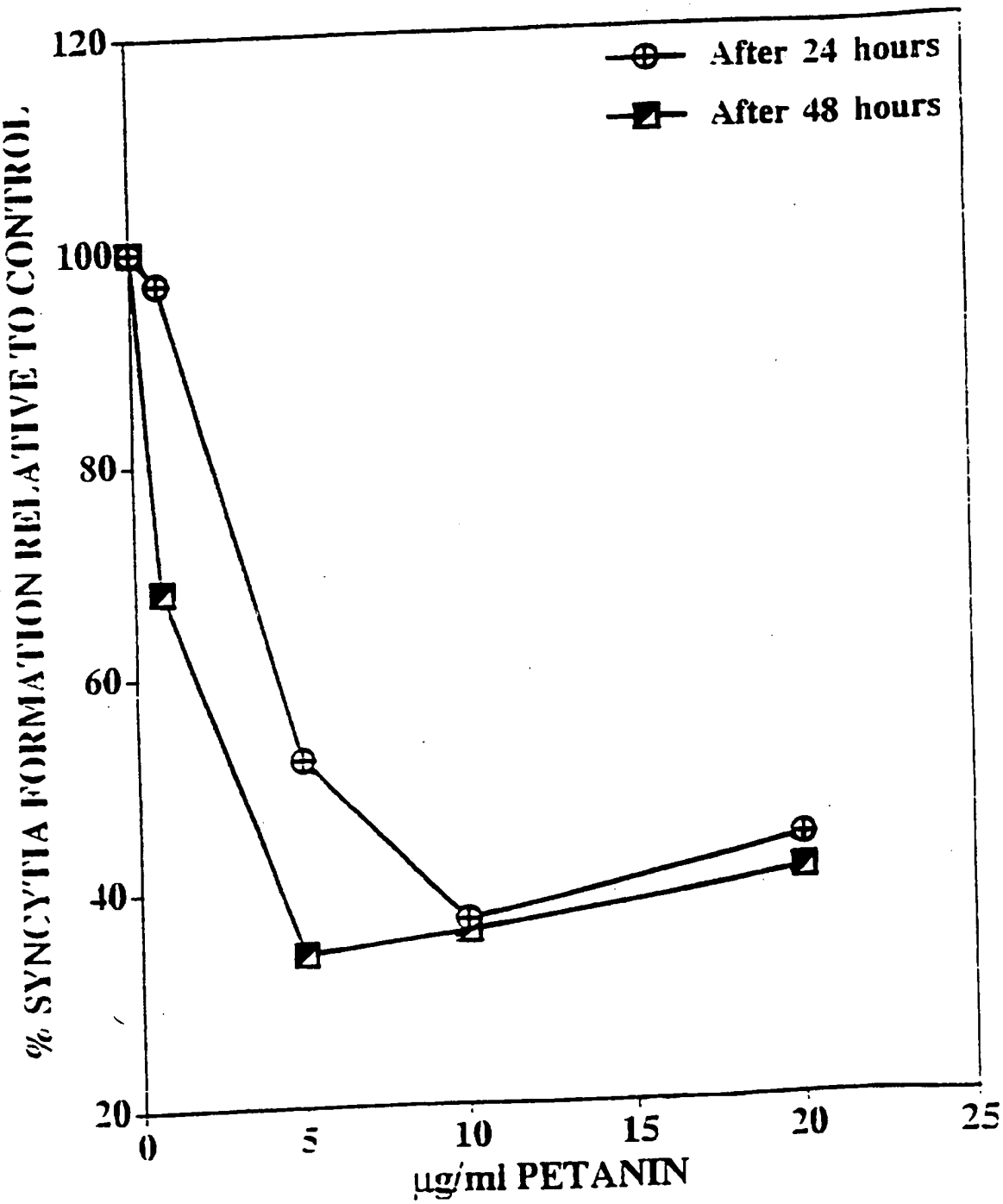

120
100
80
60
40
20
0
5
10
15
20
25
% SYNCYTIA FORMATION RELATIVE TO CONTROL
µg/ml PETANIN
After 24 hours
After 48 hours

Figure 6

i) 315 ± 15 nm ii) 520 ± 20 nm

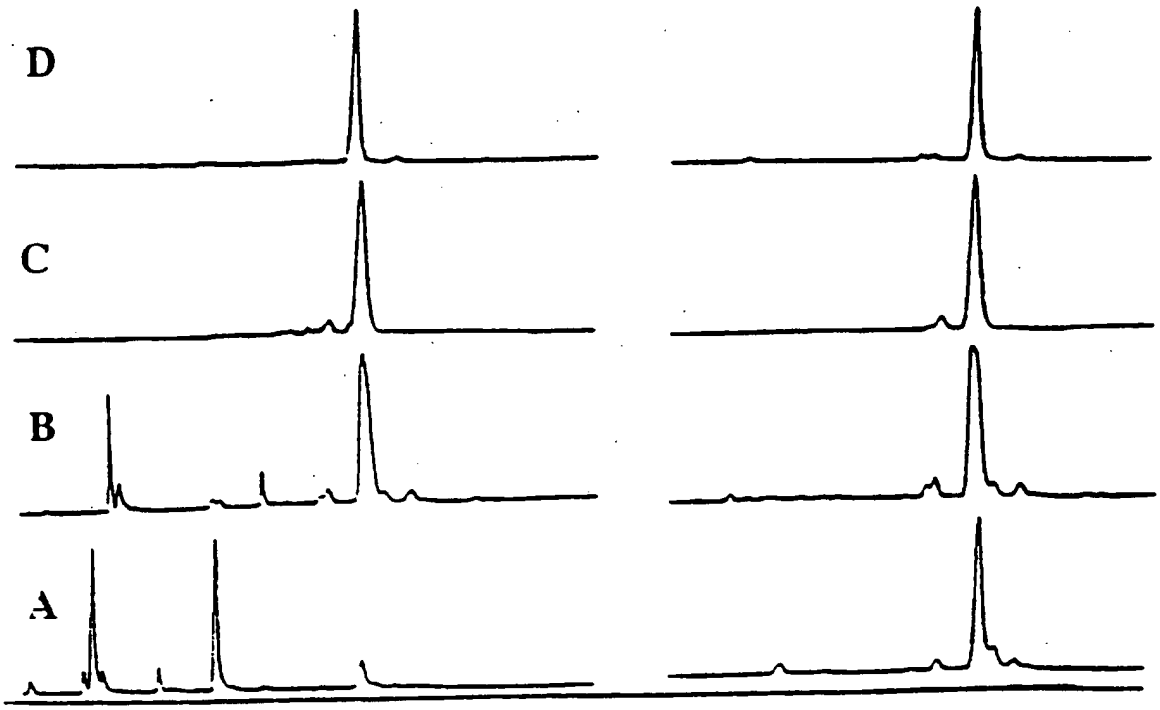


Figure 7

The structure of Petanin isolated from *Solanum tuberosum*

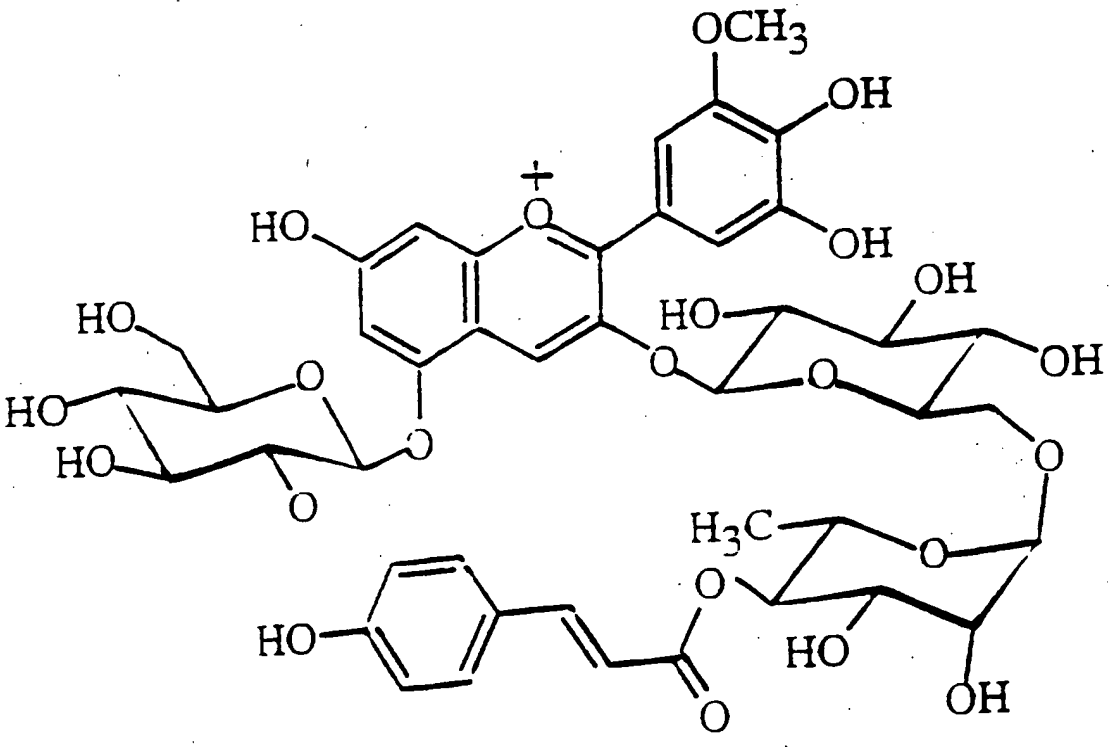


Figure 8

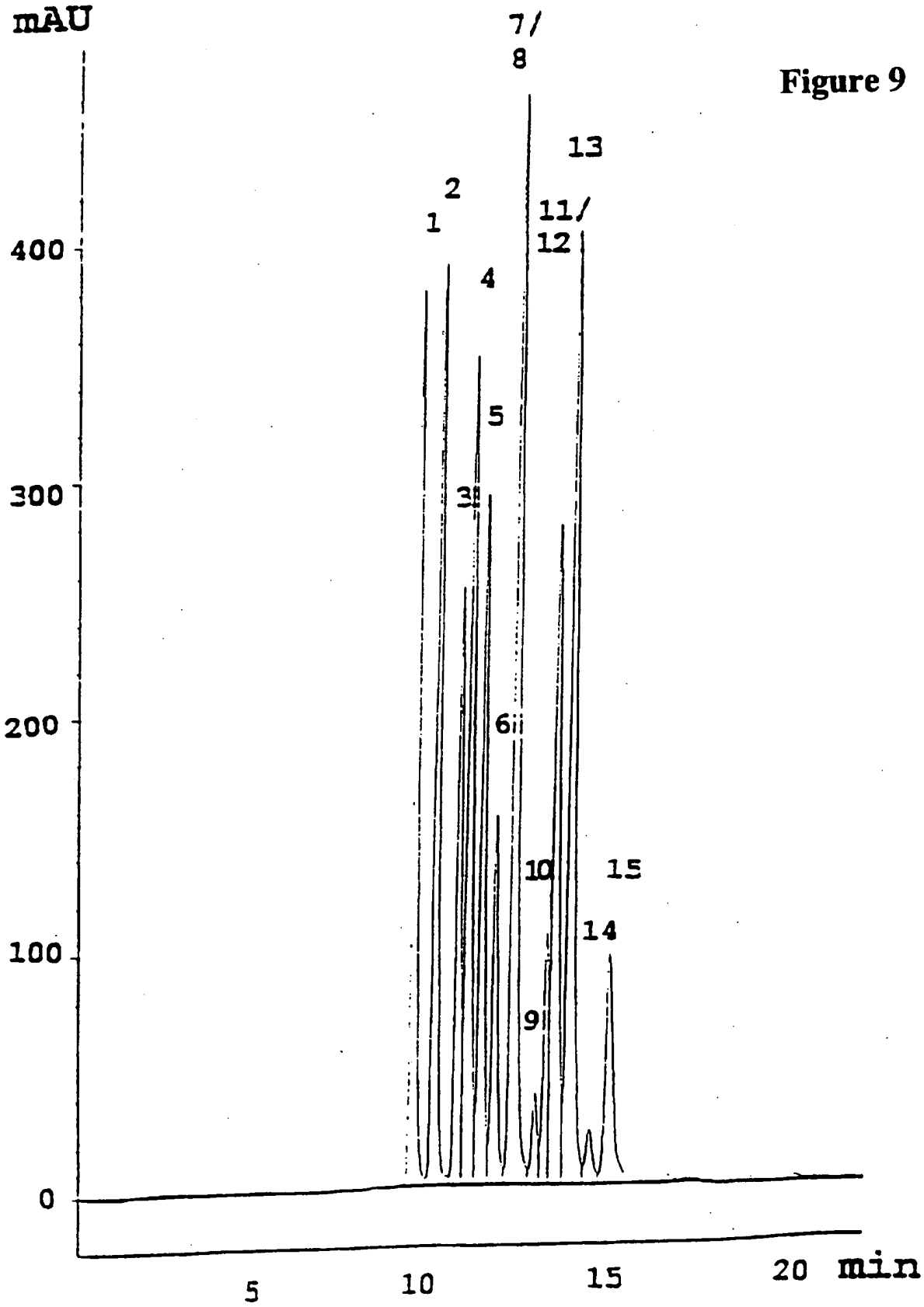
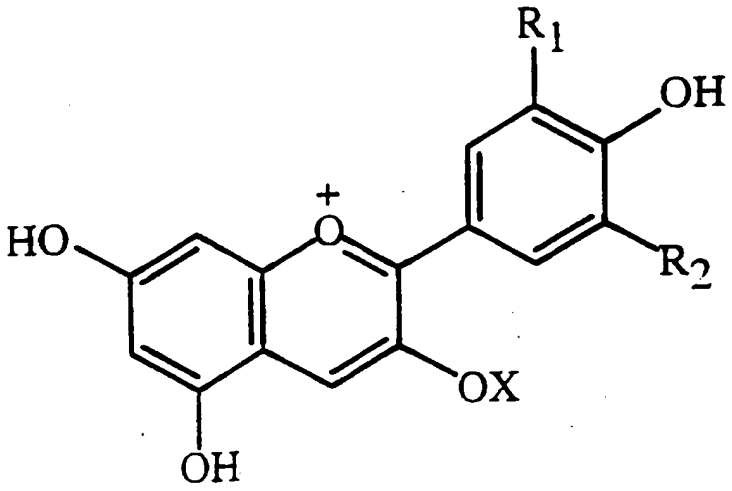


Figure 9

Structures and relative proportions (%) of the individual anthocyanins in Sample II



COMPOUND	R1	R2	X	(%)
1. Delphinidin-3-galactoside	OH	OH	galactose	10.6
2. Delphinidin-3-glucoside	OH	OH	glucose	10.7
3. Cyanidin-3-galactoside	OH	H	galactose	6.8
4. Delphinidin-3-arabinoside	OH	OH	arabinose	10.6
5. Cyanidin-3-glucoside	OH	H	glucose	8.1
6. Petunidin-3-galactoside	OCH_3	OH	galactose	4.6
7. Cyanidin-3-arabinoside	OH	H	arabinose	*
8. Petunidin-3-glucoside	OCH_3	OH	glucose	15.2*
9. Peonidin-3-galactoside	OCH_3	H	galactose	1.2
10. Petunidin-3-arabinoside	OCH_3	OH	arabinose	2.9
11. Peonidin-3-glucoside	OCH_3	H	glucose	**
12. Malvidin-3-galactoside	OCH_3	OCH_3	galactose	10.3**
13. Malvidin-3-glucoside	OCH_3	OCH_3	glucose	14.1
14. Peonidin-3-arabinoside	OCH_3	H	arabinose	0.9
15. Malvidin-3-arabinoside	OCH_3	OCH_3	arabinose	4.0

* Pigment 7 and 8 together
** Pigment 11 and 12 together

Figure 10

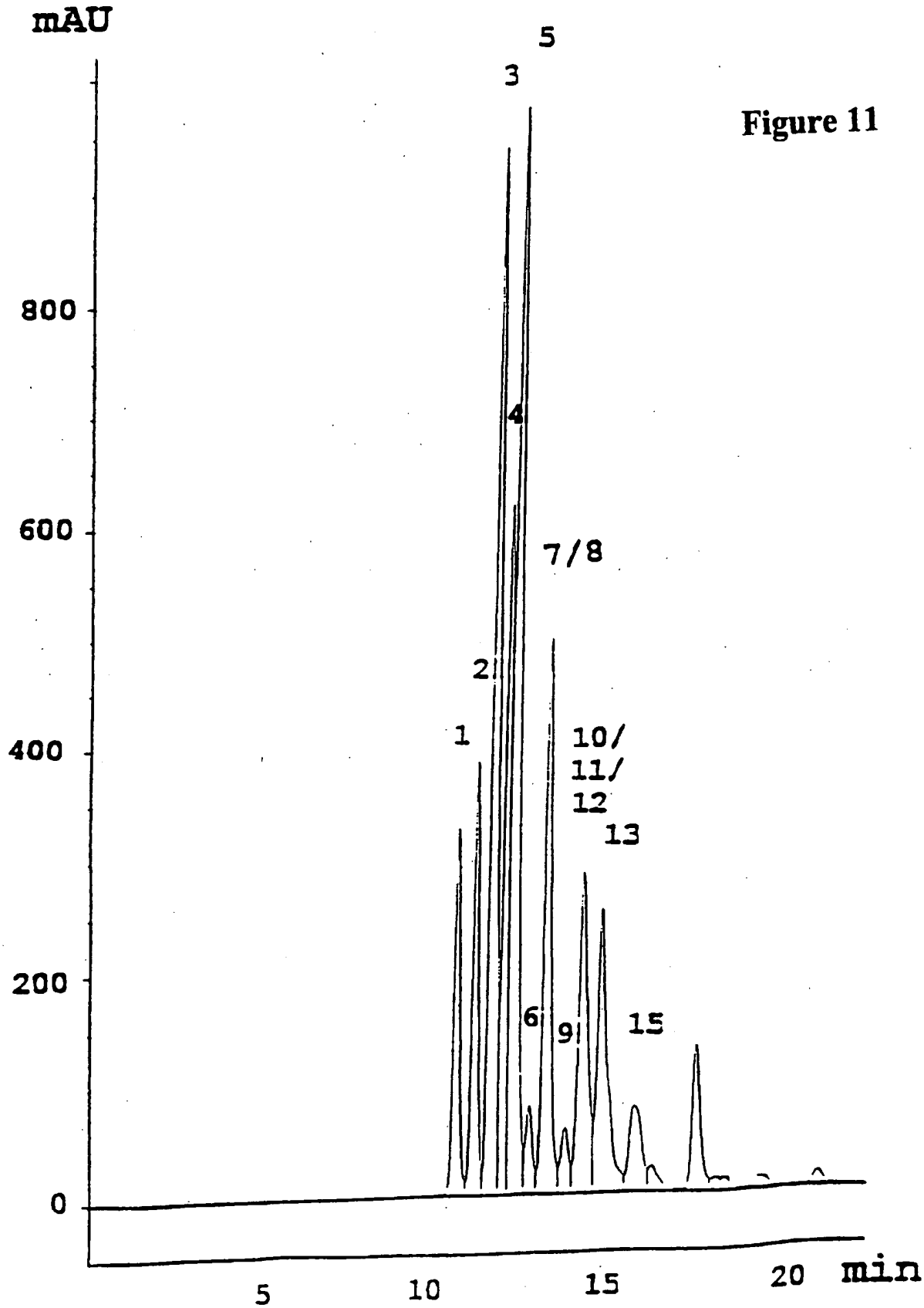
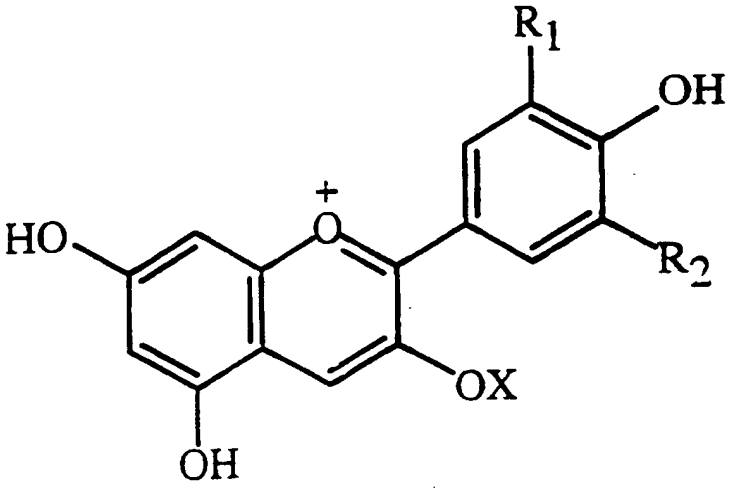


Figure 11

Structures and relative proportions (%) of the individual anthocyanins in Sample III



COMPOUND	R1	R2	X	(%)
1. Delphinidin-3-galactoside	OH	OH	galactose	6.4
2. Delphinidin-3-glucoside	OH	OH	glucose	7.4
3. Cyanidin-3-galactoside	OH	H	galactose	20.2
4. Delphinidin-3-arabinoside	OH	OH	arabinose	11.0
5. Cyanidin-3-glucoside	OH	H	glucose	22.8
6. Petunidin-3-galactoside	OCH_3	OH	galactose	1.6
7. Cyanidin-3-arabinoside	OH	H	arabinose	*
8. Petunidin-3-glucoside	OCH_3	OH	glucose	11.4*
9. Peonidin-3-galactoside	OCH_3	H	galactose	1.2
10. Petunidin-3-arabinoside	OCH_3	OH	arabinose	**
11. Peonidin-3-glucoside	OCH_3	H	glucose	**
12. Malvidin-3-galactoside	OCH_3	OCH_3	galactose	8.5**
13. Malvidin-3-glucoside	OCH_3	OCH_3	glucose	7.4
14. Peonidin-3-arabinoside	OCH_3	H	arabinose	trace
15. Malvidin-3-arabinoside	OCH_3	OCH_3	arabinose	2.1

* Pigment 7 and 8 together

** Pigment 10, 11 and 12 together

Figure 12

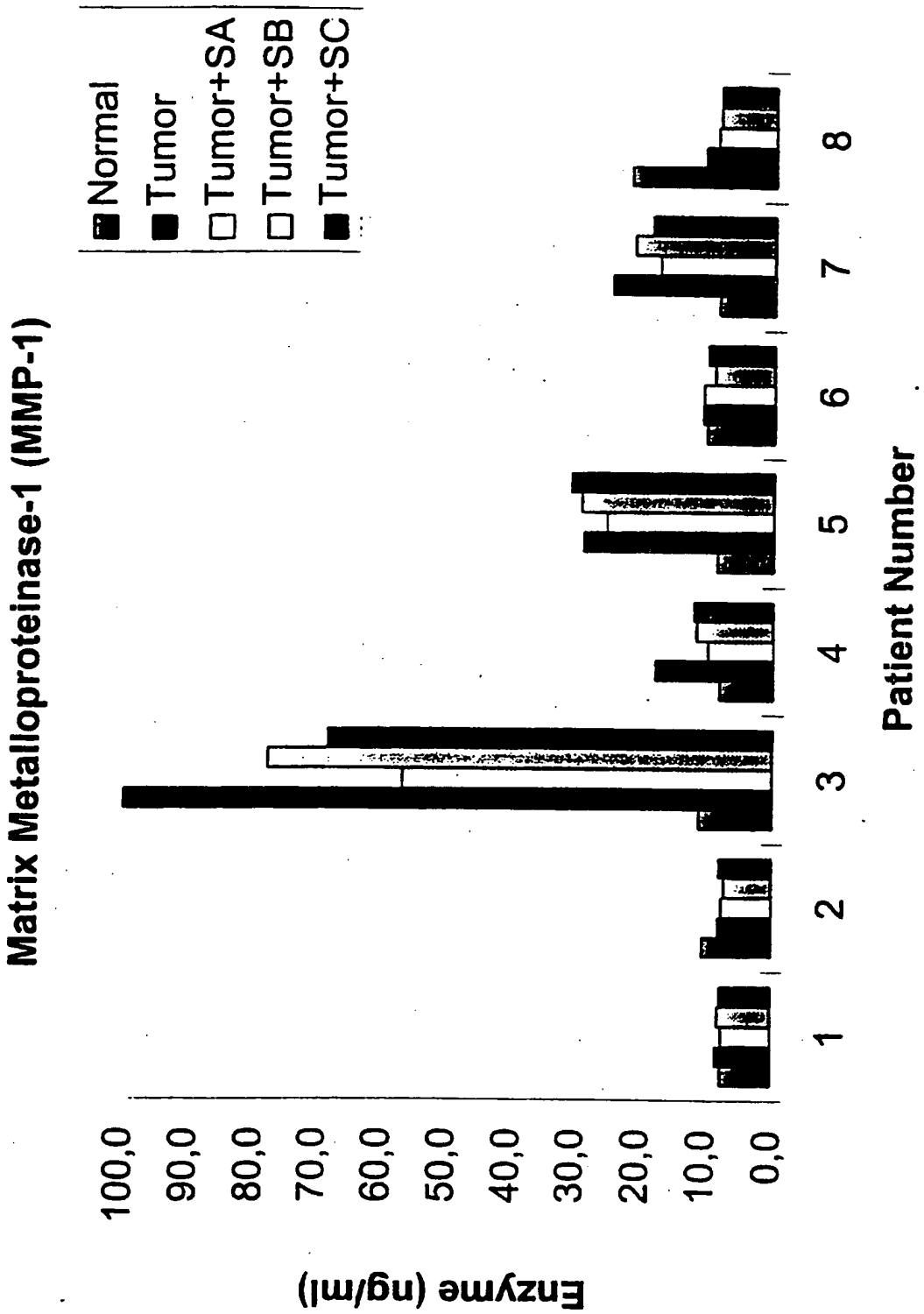

Matrix Metalloproteinase-1 (MMP-1)
Normal
Tumor
Tumor+SA
Tumor+SB
Tumor+SC
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
Enzyme (ng/ml)
1
2
3
4
5
6
7
8
Patient Number

Figure 13

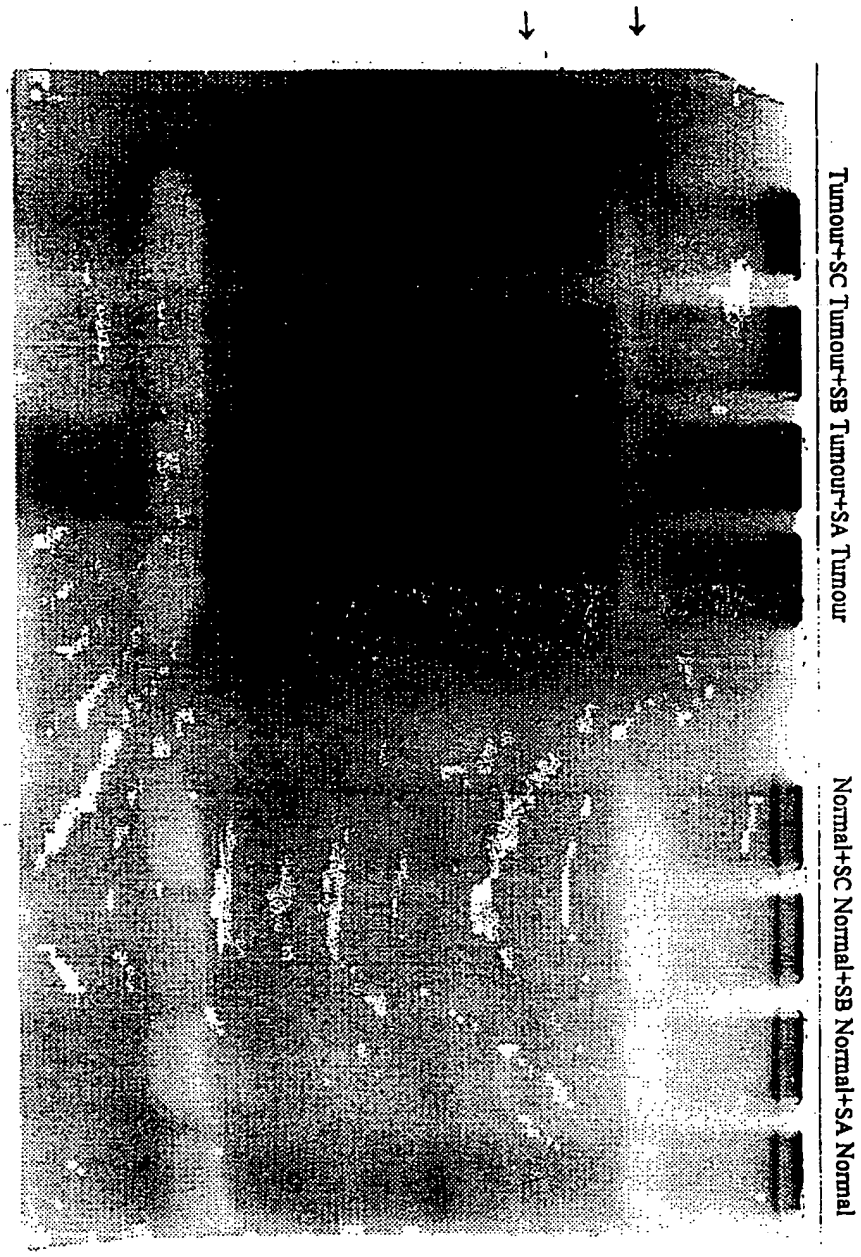

Rectum
Tumour+SC Tumour+SB Tumour+SA Tumour
Normal+SC Normal+SB Normal+SA Normal

Figure 14

Figure 15


Normal
Normal
Tumour
Tumour+SA
Tumour+SB
Tumour+SC
Ventricle

Figure 16

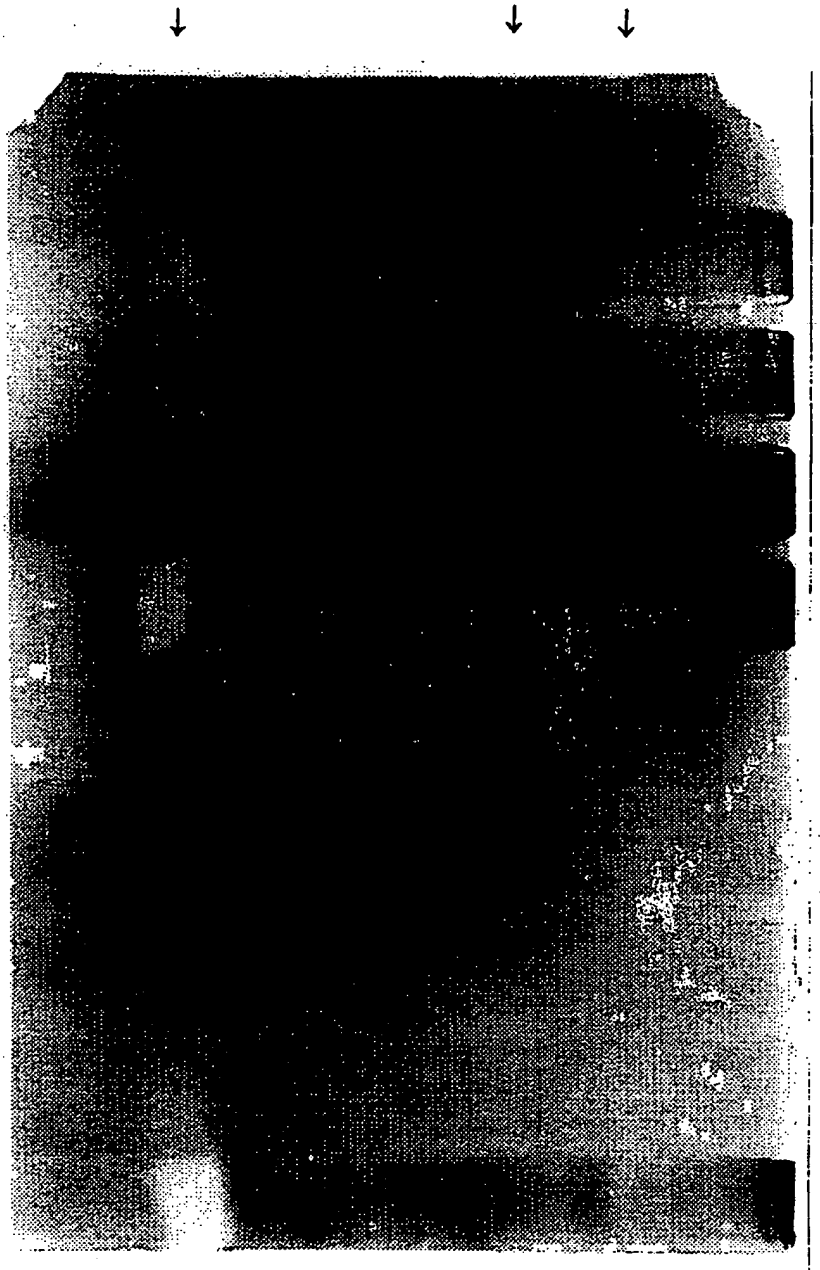

Pancreas
Tumour+SC Tumour+SB Tumour+SA Tumour
Normal+SC Normal+SB Normal+SA Normal

Figure 17

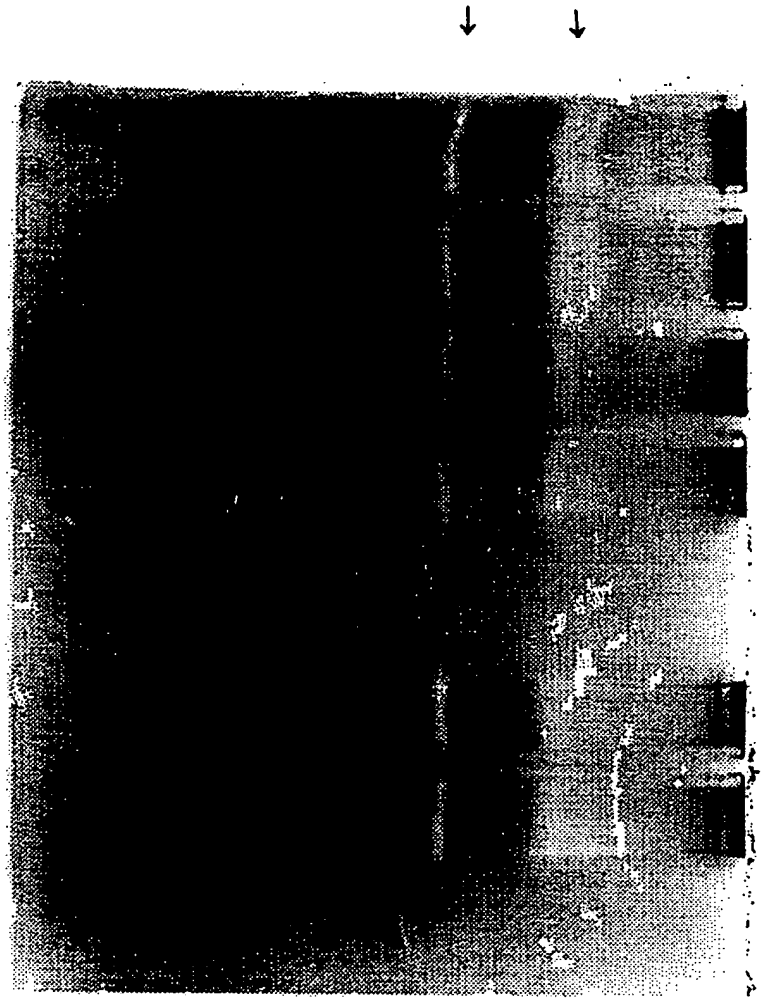


Figure 18

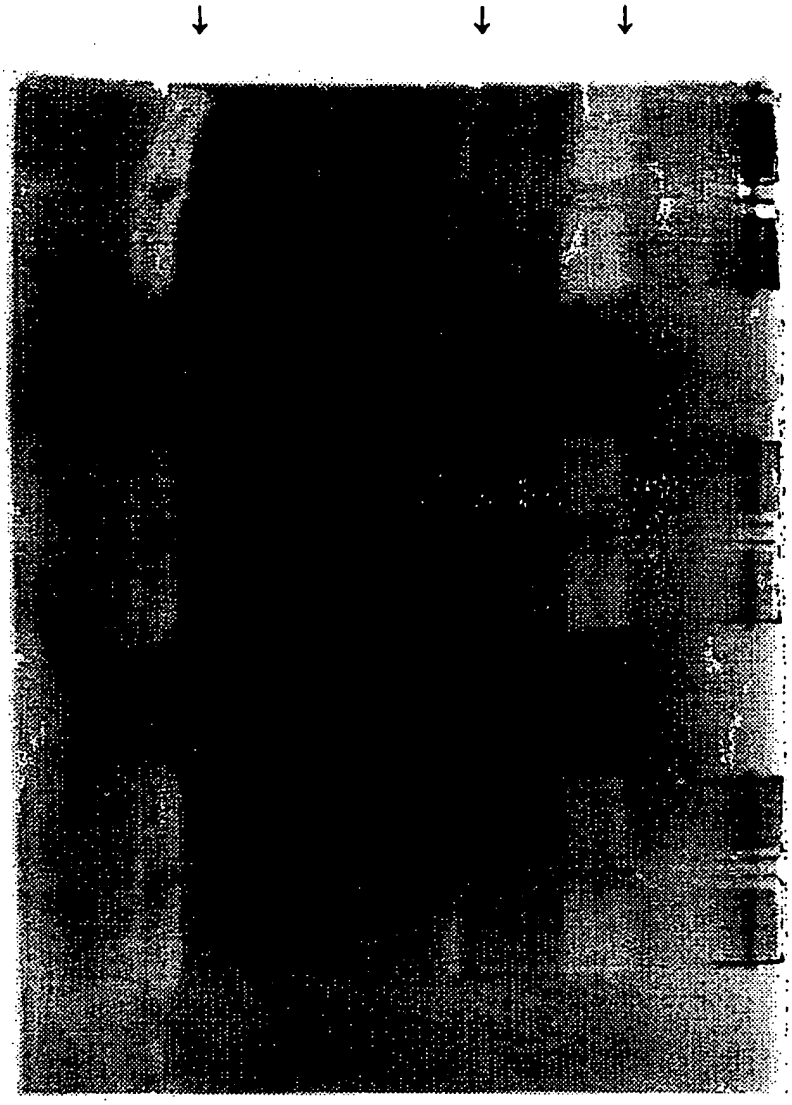


Figure 19

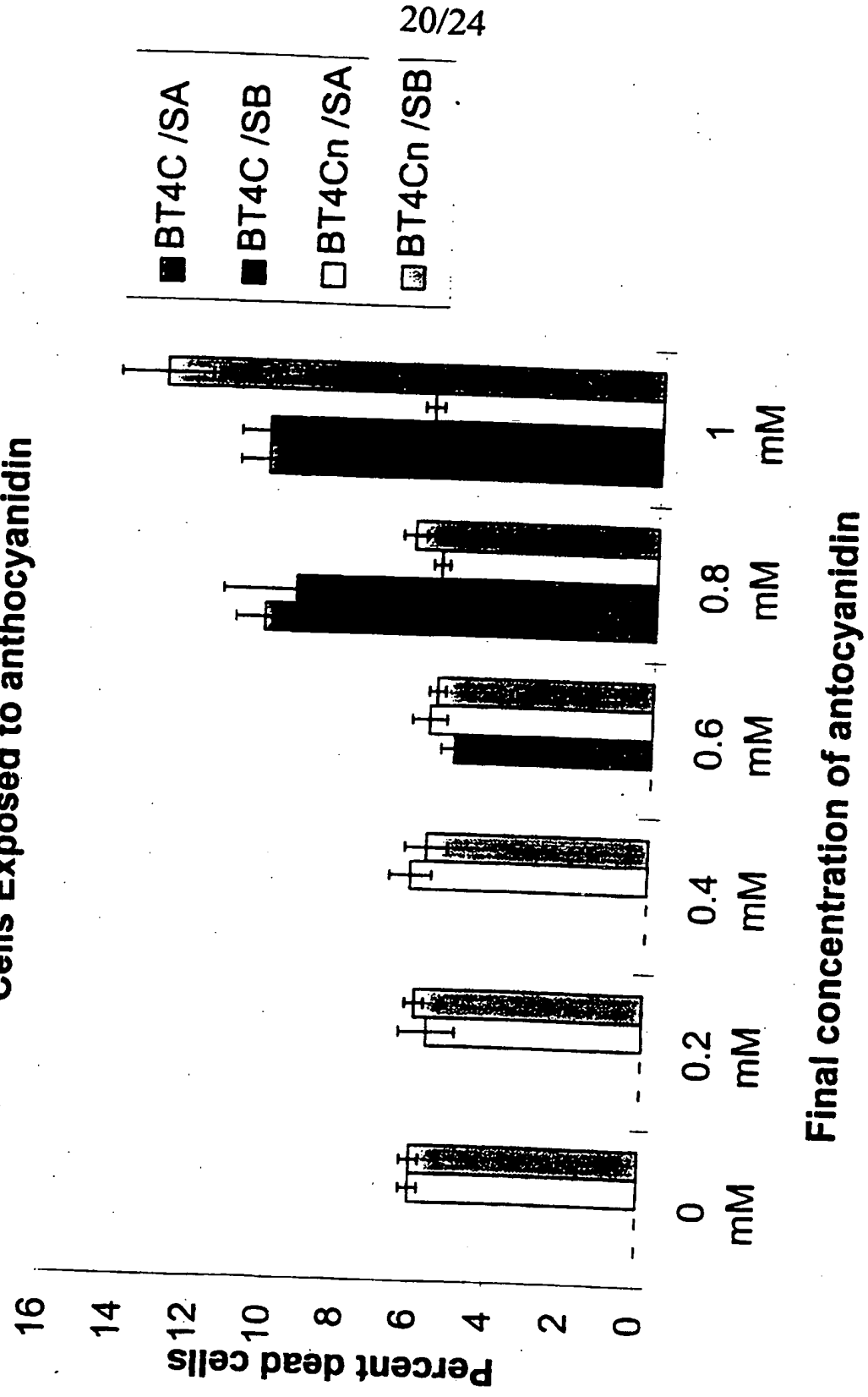

BT4C /SA
BT4C /SB
BT4Cn /SA
BT4Cn /SB
Percent dead cells
0
2
4
6
8
10
12
14
16
Cells Exposed to anthocyanidin
0 mM
0.2 mM
0.4 mM
0.6 mM
0.8 mM
1 mM
Final concentration of antocyanidin

Figure 20

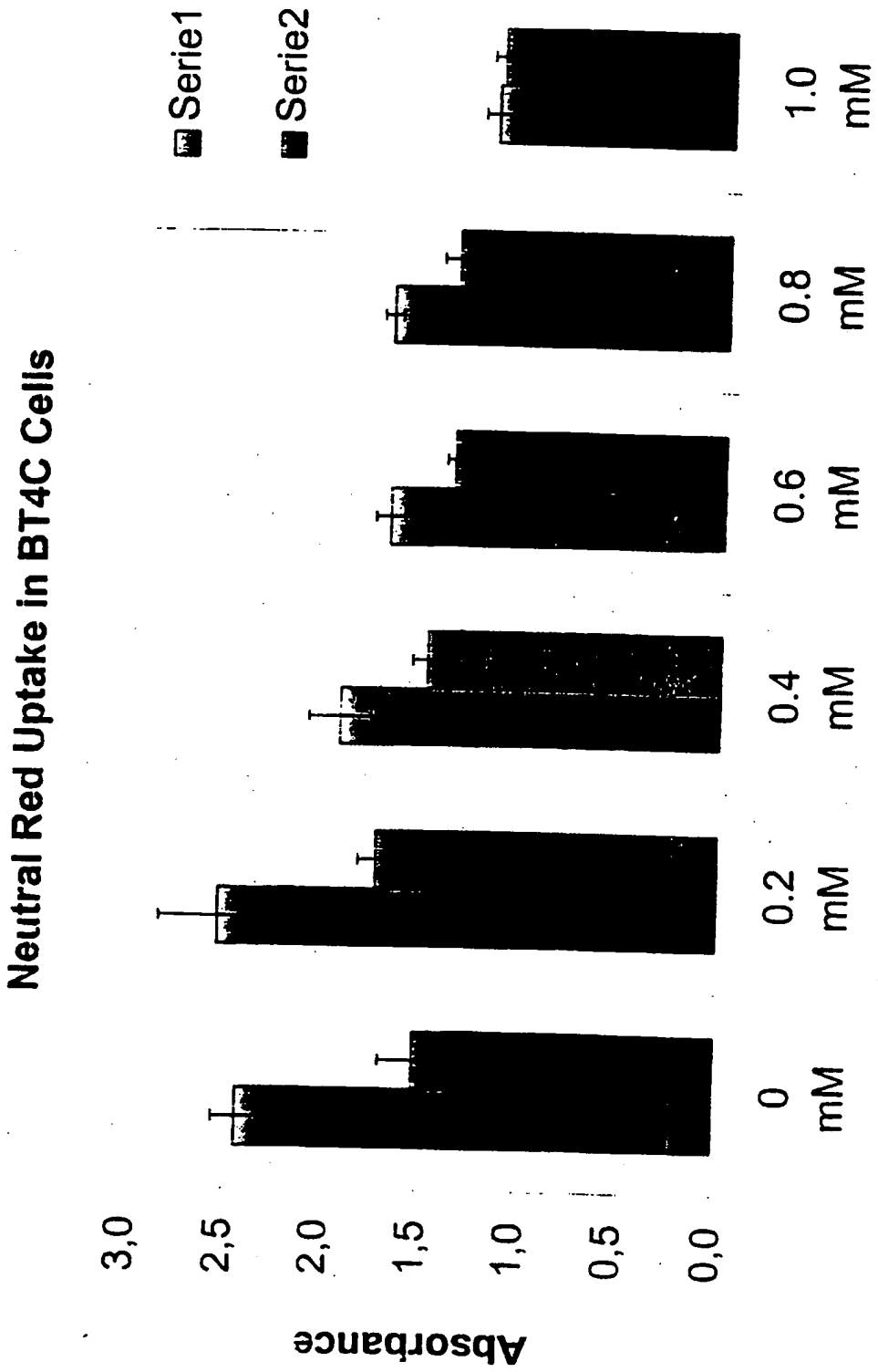

Neutral Red Uptake in BT4C Cells
Absorbance
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Serie1
Serie2
0 mM
0.2 mM
0.4 mM
0.6 mM
0.8 mM
1.0 mM
Final concentration of anthocyanidin

Figure 21

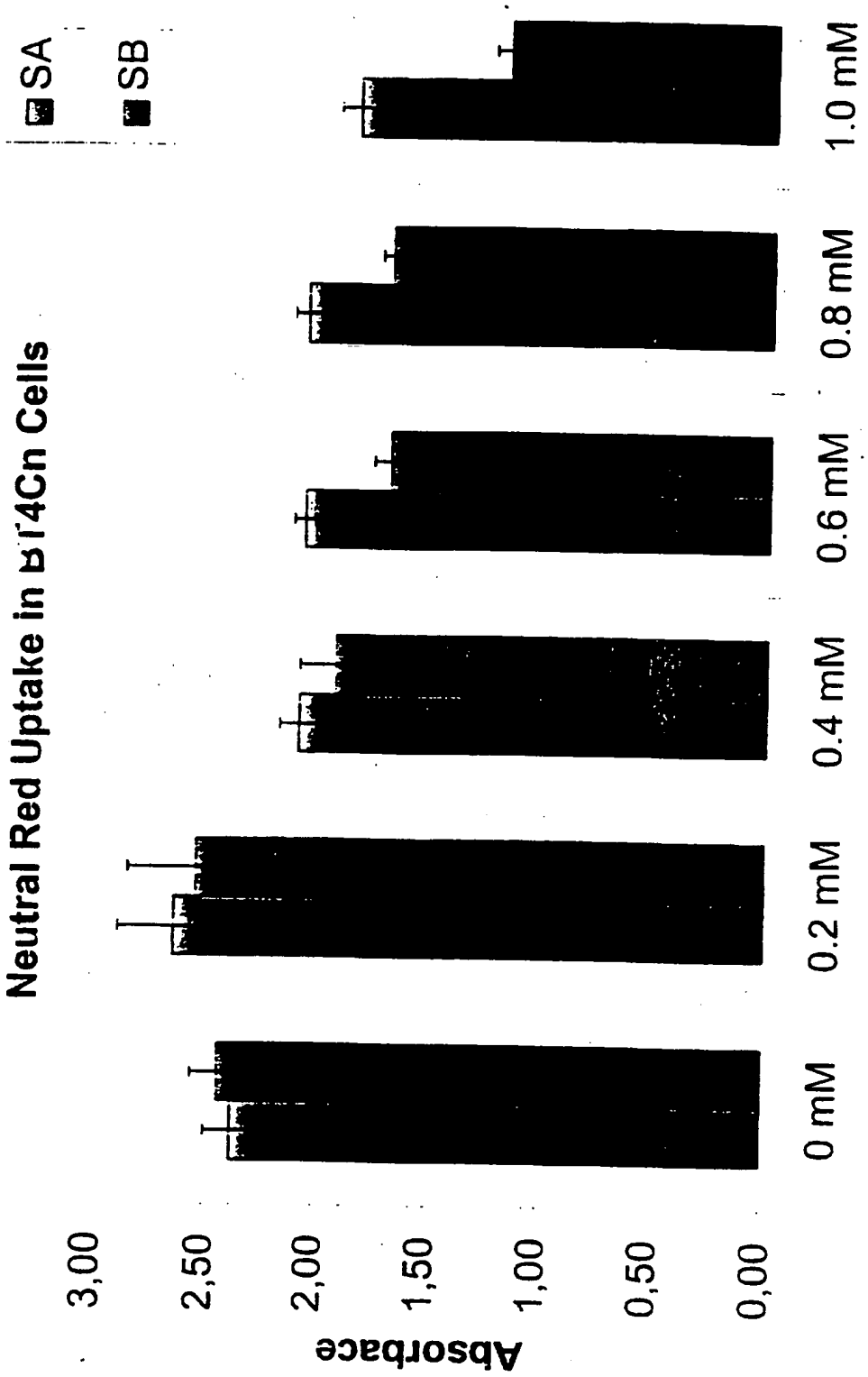

Neutral Red Uptake in BT4Cn Cells
SA
SB
Absorbace
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0 mM
0.2 mM
0.4 mM
0.6 mM
0.8 mM
1.0 mM
Final concentration of anthocyanidin

Figure 22

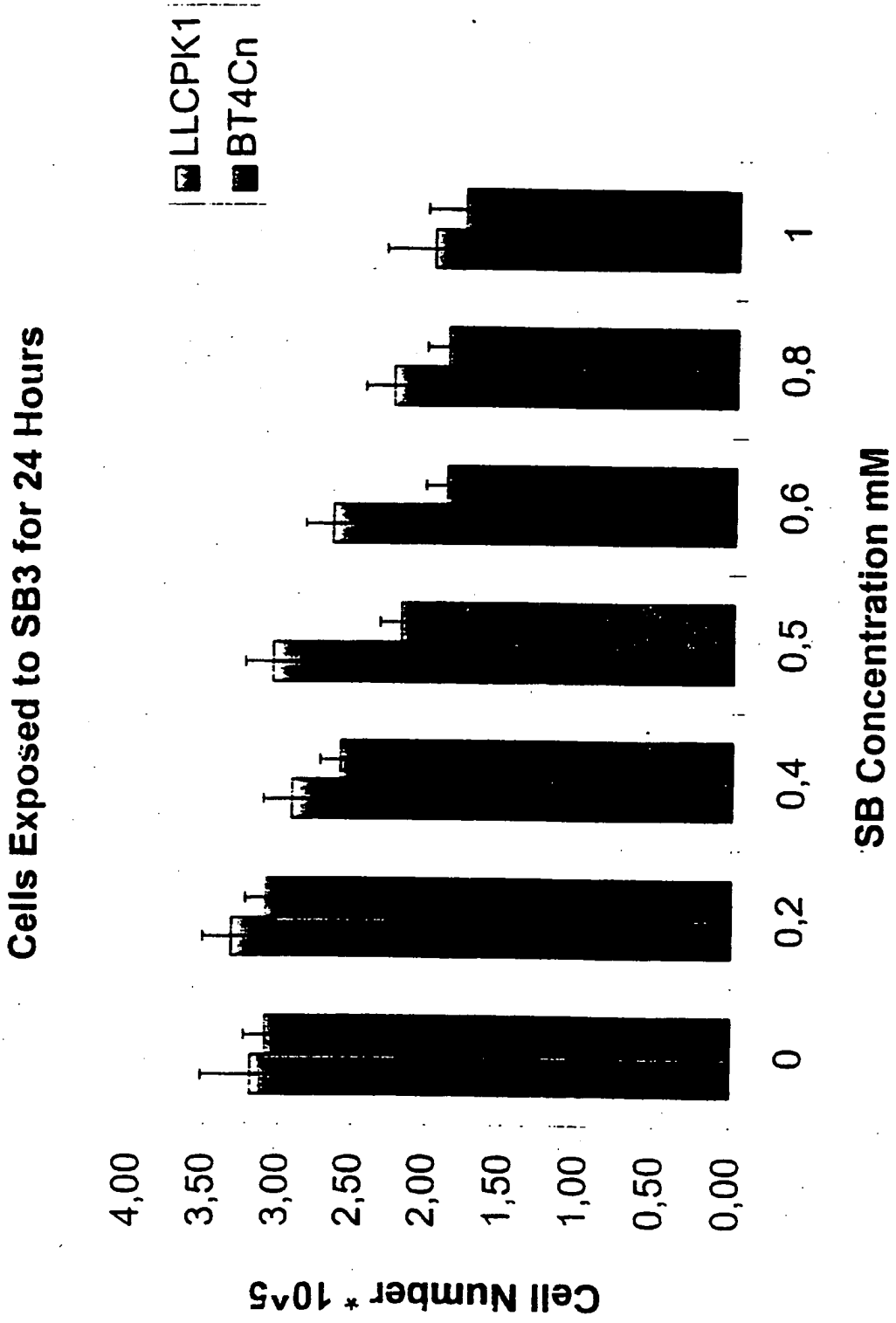

Cells Exposed to SB3 for 24 Hours
LLCPK1
BT4Cn
Cell Number * 10^5
4,00
3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
0
0,2
0,4
0,5
0,6
0,8
1
SB Concentration mM

Figure 23

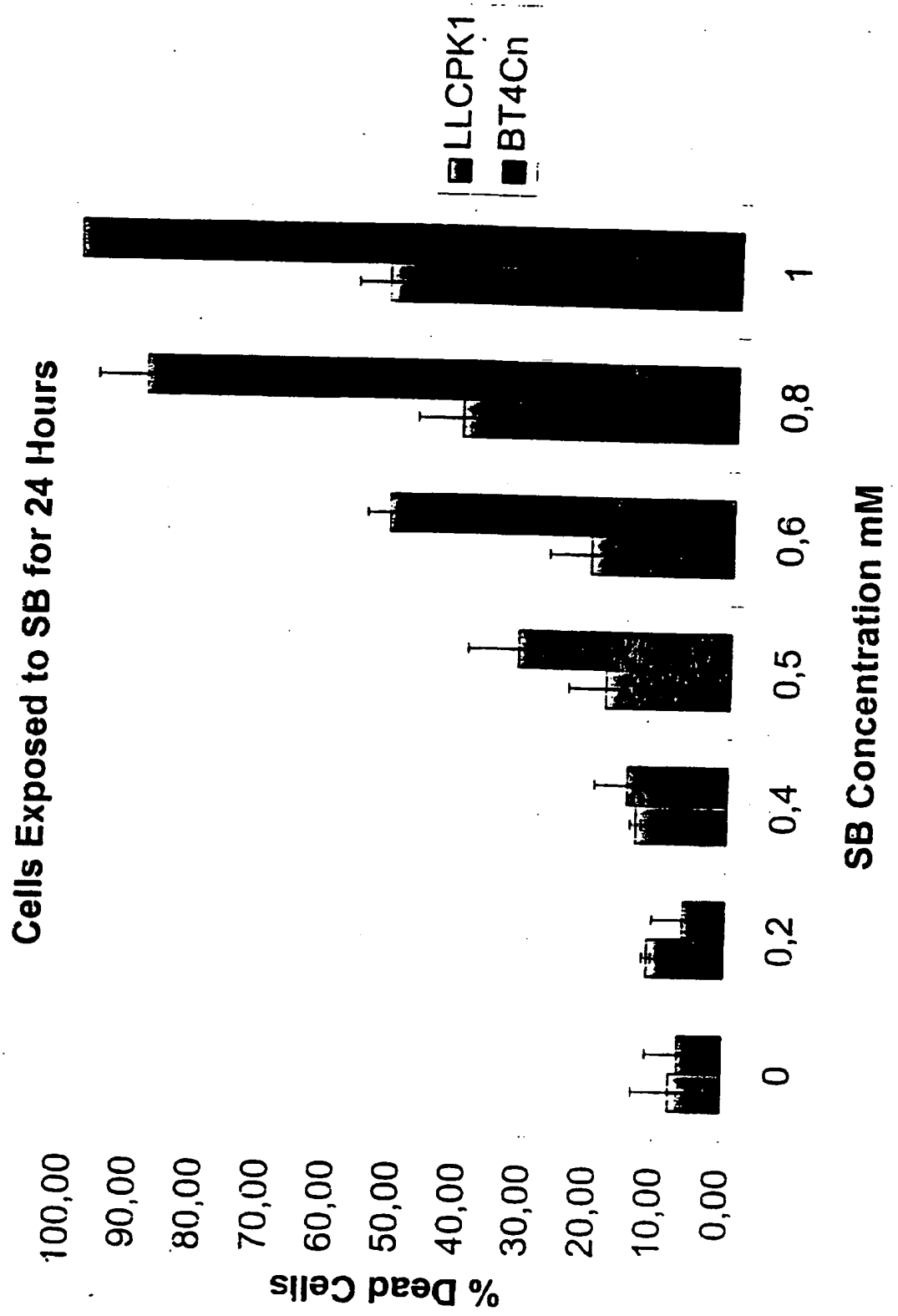

Cells Exposed to SB for 24 Hours
% Dead Cells
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
LLCPK1
BT4Cn
0
0,2
0,4
0,5
0,6
0,8
1
SB Concentration mM

Figure 24

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NO 97/00100

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H17/065 C07D311/62 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 015, no. 484 (C-0892), 9 December 1991 & JP 03 209321 A (TORAY IND INC), 12 September 1991, see abstract ---	1-4,6-8, 10, 13-15, 19,22, 26,28, 32,33
X	PATENT ABSTRACTS OF JAPAN vol. 015, no. 484 (C-0892), 9 December 1991 & JP 03 209320 A (TORAY IND INC), 12 September 1991, see abstract ---	1-8, 13-15, 19,22,26
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 September 1997	06 -10- 1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Day, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NO 97/00100

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 015, no. 484 (C-0892), 9 December 1991 & JP 03 209319 A (TORAY IND INC), 12 September 1991, see abstract	1-5,12, 15,19, 22,26
X	PATENT ABSTRACTS OF JAPAN vol. 006, no. 214 (C-131), 27 October 1982 & JP 57 120584 A (KANEBO KK), 27 July 1982, see abstract	1,9
X	PATENT ABSTRACTS OF JAPAN vol. 006, no. 214 (C-131), 27 October 1982 & JP 57 118580 A (KANEBO KK), 23 July 1982, see abstract	1,9
X	PATENT ABSTRACTS OF JAPAN vol. 005, no. 067 (C-053), 7 May 1981 & JP 56 016412 A (KANEBO LTD), 17 February 1981, see abstract	1,9
X	PATENT ABSTRACTS OF JAPAN vol. 017, no. 052 (C-1022), 2 February 1993 & JP 04 264027 A (TAIYO KAGAKU CO LTD), 18 September 1992, see abstract	1,9
A	EP 0 412 300 A (INVERNI DELLA BEFFI S.P.A) 13 February 1991 see column 3; examples	1,24,25, 31
A	US 4 999 423 A (EIICHI IDEKA) 12 March 1991. see claims	15-19, 22,23
P,X	WO 96 11692 A (UNIFOB STIFTELSEN UNIVERSITETSFORSKNING BERGEN) 25 April 1996 see the whole document	1,28,32
P,X	PATENT ABSTRACTS OF JAPAN vol. 096, no. 008, 30 August 1996 & JP 08 104628 A (SUMITOMO PHARMACEUT CO LTD), 23 April 1996, see abstract	1,11,28, 32

6

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00100

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 32 and 33 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

Claims 27, 30, 31
See annex

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 27,30,31

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The scope of the independent claim 27, and thus also the dependent claim 30 and 31 cannot be determined, since they are defined (limited) by reference to a number of non patent publications, the contents of which (in particular compounds disclosed therein) form a disclaimer to said claims. As such, it is the opinion of the ISA (Art. 17 PCT) that a meaningful search cannot be performed for these claims.

INTERNATIONAL SEARCH REPORT

....ormation on patent family members

International Application No
PCT/NO 97/00100

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